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14. ABSTRACT In the past year, our laboratory has made significant strides towards the translation of observations made in the laboratory into clinical strategies that would help patients with prostate cancer. At the time of the last report, in a high profile article in Cancer Research, we had shown that ErbB3 is upregulated in prostate cancer cells undergoing androgen ablation, and this upregulation may be a major cause of the progression of prostate cancer to castration resistance. Now, using cell lines and animal models, we show that pharmaceutical inhibition of ErbB3 - achieved by dual inhibition of its binding partners EGFR and ErbB2, induce apoptosis and prevent survival to castration resistance. This paper has been very well received by the scientific community and has been featured at various websites. Another article - a review on ErbB3 in prostate cancer - has also been published. We are now preparing a fourth manuscript - one that describes Nrpd1 as a transcriptional target of AR, and shows that the AR binds to the proximal Nrpd1 promoter and promotes its transcription, only in castration sensitive, and not in castration resistant cells. This would complete four of the 5 tasks proposed in the grant.					
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INTRODUCTION: Patients with advanced prostate cancer (PCa) are initially susceptible to androgen withdrawal therapy (AWT), but ultimately develop resistance to this therapy (castration-resistant PCa, CRPC). The treatment options for patients who fail AWT are limited; hence the long-term goal of these studies is to identify therapeutic strategies to prolong the effectiveness of AWT. The ErbB receptor tyrosine kinase (RTK) family regulates proliferation and survival in PCa. Multiple studies suggested that ErbB3 plays a role in promoting PCa, however, its mechanism of action and the pathways mediating its effects were unknown. Hence, we investigate the role of ErbB3 in PCa progression.

BODY:

Specific Aim 1. To test the hypothesis that increased ErbB3 during androgen ablation results in androgen independence of prostate cancer cells.

***Task 1:** We will examine in paraffin embedded prostate cancer tissues (human anatomical samples) whether there is increased ErbB3 and decreased Nrdp1 expression in androgen independent tumors from human patients. (Months 1-9)*

Complete – report in previous Annual Report (2010).

***Task 2:** In an animal model of prostate cancer progression, we will investigate whether inhibition of ErbB3 during androgen ablation prevents the development of CRPC tumors. (Months 9-18)*

Partial report in previous Annual Report (2010). In that report, we showed that androgen ablation caused an increase in ErbB3 levels in the CWR22 mouse xenograft model of prostate cancer.

In the past year, we continued our investigation of the effect of inhibition of ErbB3 at the time of androgen ablation. Since ErbB3 inhibitors are not in clinical use at the current time, we devised an innovative method to pharmacologically inhibit ErbB3 with currently FDA approved drugs. Since ErbB3 requires heterodimerization with either EGFR or ErbB2 for activation, we showed that dual inhibition of EGFR by erlotinib (Tarceva) and ErbB2 by trastuzumab (Herceptin) also inhibited ErbB3 activation (explained in a review article included in **Appendix A**). Hence, dual application of an EGFR inhibitor and an ErbB2 inhibitor caused a regression of tumors, together with an inhibition of ErbB3 (shown in **Appendix B**).

This article is still in press; however, an electronic version is already available for preview at the journal site. Based on this preview version, multiple websites have already featured the findings of this study: It has been featured on MDLinx (<http://www.mdlinx.com/urology/news-article.cfm/3717562>), Silobreaker (http://www.silobreaker.com/dual-egfrher2-inhibition-sensitizes-prostate-cancer-cells-to-androgen-withdrawal-by-suppressing-erbb3-5_2264783756445352038), and in Affective (<http://affective.com/news/dual-egfrher2-inhibition-sensitizes-prostate-cancer-cells-to-androgen-withdrawal-by-suppressing-erbb3>) as an article in Urology that matters in the daily lives of physicians and other healthcare professionals.

Specific Aim 2. To test the hypothesis that Nrdp1 mediates the regulation of ErbB3 expression by the androgen receptor in androgen dependent cells, but this regulation is lost in androgen independence.

***Task 3:** We will identify a role for Nrdp1 in the expression of ErbB3 during androgen withdrawal and in androgen independence. (Months 1-12)*

Complete – report in previous Annual Report (2010)

***Task 4:** In androgen dependent cells we will determine how the androgen receptor regulates Nrdp1 transcription. (Months 13-24)*

Outcomes: A fourth manuscript on this topic is being planned, and the key features of this article are presented here.

ErbB3 stimulates cell growth and is overexpressed in castration resistant prostate cancer. Using androgen dependent LNCaP cells and its castration resistant subline C4-2 (**Figure 1A**), our lab has shown that ErbB3 is overexpressed in castration resistant prostate cancer (CRPC) (**Figure 1B**). In addition, ectopic expression of ErbB3 in LNCaP cells, significantly increased cell proliferation (**Figure 1C**). This data led us to hypothesize that ErbB3 is a significant factor in PCa progression, and that elucidation of the mechanism of its regulation in PCa would help design improved therapeutic modules that prevent CRPC and improve patient survival.

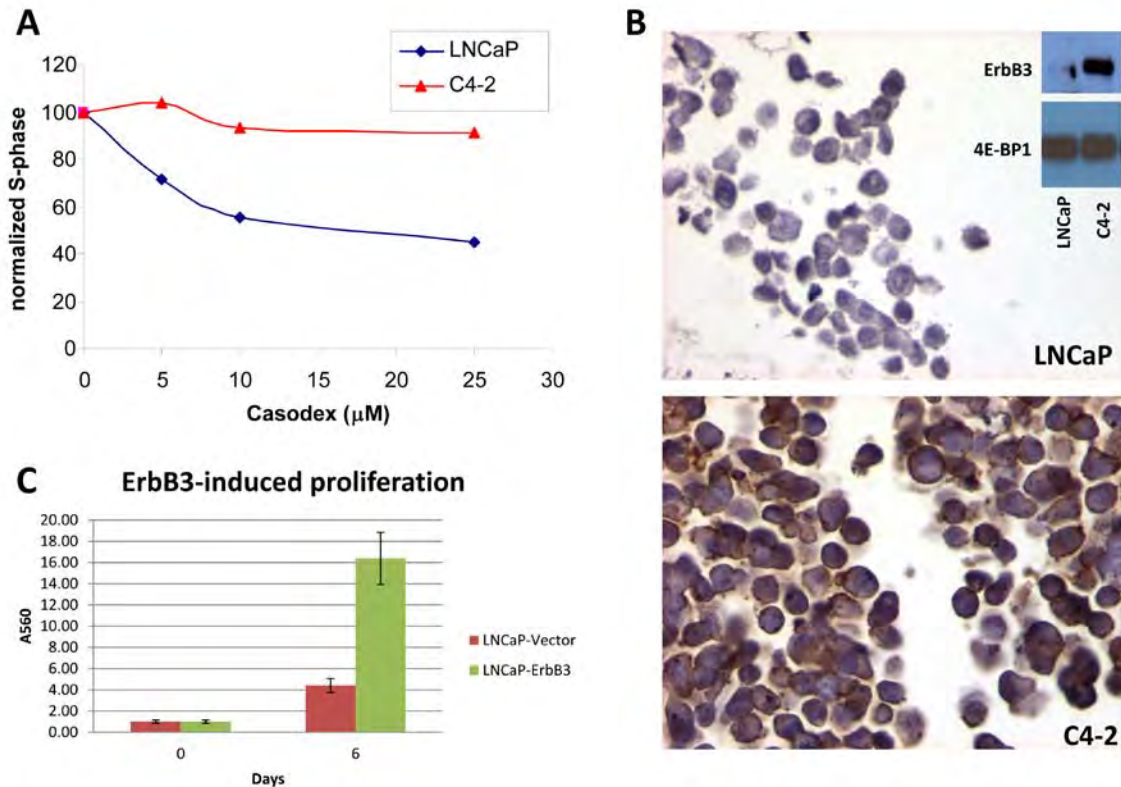


FIGURE 1

Figure 1. ErbB3 stimulates cell growth and androgen independence in prostate cancer. (A) Flow analysis on LNCaP and C4-2 cells with increasing doses of Casodex. LNCaP and C4-2 cells were grown in FBS medium and treated with either 5uM, 10uM, or 25 uM of Casodex on day 2. Casodex inhibits AR transcriptional activity and increasing doses reduces the cell proliferation of LNCaP cells but not C4-2, indicating that LNCaP cells are androgen dependent but C4-2 cells are androgen independent. (B) Immunohistochemistry for anti-ErbB3 in LNCaP and C4-2 cells. C4-2 cells show increased ErbB3 at the cell membrane indicating increased activity. Cell lysates blotted for anti-ErbB3 and anti-4E-BP1 show the increased ErbB3 expression in C4-2 compared to LNCaP cells. (C) Flow analysis of LNCaP cells transfection with either control vector or ErbB3-overexpression vector. LNCaP cells were grown in FBS medium and transfected with control or ErbB3 vector and allowed to grow for 6 days. On the first day cell proliferation was the same for control and ErbB3, while after 6 days the increased ErbB3 significantly increased cell proliferation.

Nrdp1 transcription is androgen-regulated in LNCaP, but not in C4-2 cells. Previous studies showed that ErbB3 is down regulated by the E3 ubiquitin ligase Nrdp1 in prostate cancer cells (Liquin's reference), so we investigated the mechanism of regulation of Nrdp1 expression in this system. In addition, we previously showed that the expression of Nrdp1 is androgen-regulated (Liquin's reference) Analysis of the Nrdp1 genomic annotation revealed that there are two promoters that contain three androgen response elements (ARE) in the Nrdp1 gene region (**Figure 2A**). Of the three AREs, one (ARE3) is located in the promoter upstream of the transcriptional start site and is a full ARE with two palindromic sequences similar to the PSA AREs, while the other two (ARE1 and ARE2) are located within the internal promoter and contain only one of the palindromic sequences (**Figure 2A**). Based on this it would indicate that the AR would have stronger, more specific binding to ARE3 and only weaker binding to ARE1 and ARE2 and we propose that AR binds to one of the three AREs to upregulate Nrdp1 and decrease ErbB3 expression.

Comparison of Nrdp1 expression in different cell lines showed that Nrdp1 is androgen-regulated in LNCaP, but not in C4-2, cells. Western blotting in LNCaP and C4-2 cells showed that Nrdp1 protein levels were significantly lower in C4-2 compared with LNCaP (**Figure 2B**). In addition, Nrdp1 expression was higher in the presence of androgens (medium containing fetal bovine serum, FBS, and in the presence of dihydrotestosterone (DHT), compared to medium with charcoal stripped serum (CSS), indicating Nrdp1 expression was androgen dependent in LNCaP cells (**Figure 2C**).

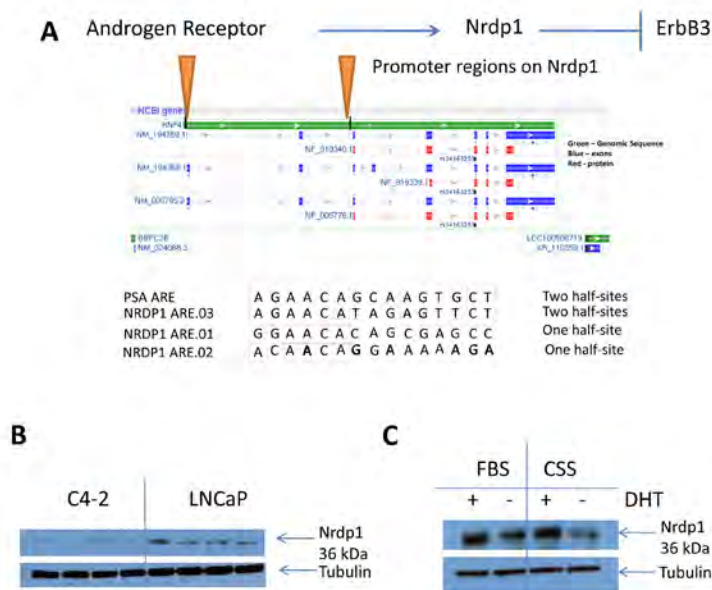


Figure 2. Nrdp1 regulation by AR in LNCaP vs C4-2 cells. (A) Proposed scheme of regulation of ErbB3 by Nrdp1 and AR. Inspection of Nrdp1 genomic annotation led to the discovery of two promoters, represented by the two arrows. Comparison of PSA and Nrdp1 AREs shows that the PSA ARE and Nrdp1 ARE3 both contain a full palindromic ARE containing two half sites, while Nrdp1 ARE1 and ARE2 both contain only one half site. (B) Nrdp1 protein levels correspond with androgen dependence. C4-2 and LNCaP cells were grown in FBS medium. Cell lysates were immunoblotted with anti-Nrdp1 and anti-tubulin antibodies. (C) Nrdp1 levels correspond with the addition of DHT. LNCaP cells were cultured in FBS or CSS medium in the presence or absence of DHT after the first day. Cell lysates were immunoblotted with anti-Nrdp1 and anti-tubulin antibodies.

Differential regulation of Nrdp1 by androgens in different castration resistant prostate cancer cell lines. We compared CWR-R1 (R1) and CWR22Rv1 (Rv1) cells, two cell lines generated from relapsed CWR22 tumors in castrated nude mice, which contain two mutant AR alleles: a full-length AR with a point mutation at H874Y, and a truncated AR that lacks the ligand binding domain. Both cell lines express the 36 kDa isoform of Nrdp1, but only R1 cells express ErbB3, whereas Rv1 cells express the 28 kDa Nrdp1 isoform (**Figure 3A**). In addition, the AR in R1 cells seems to regulate Nrdp1 in an androgen dependent manner, while Nrdp1 expression in Rv1 cells is not regulated by the presence or absence of androgens (**Figures 3B and C**).

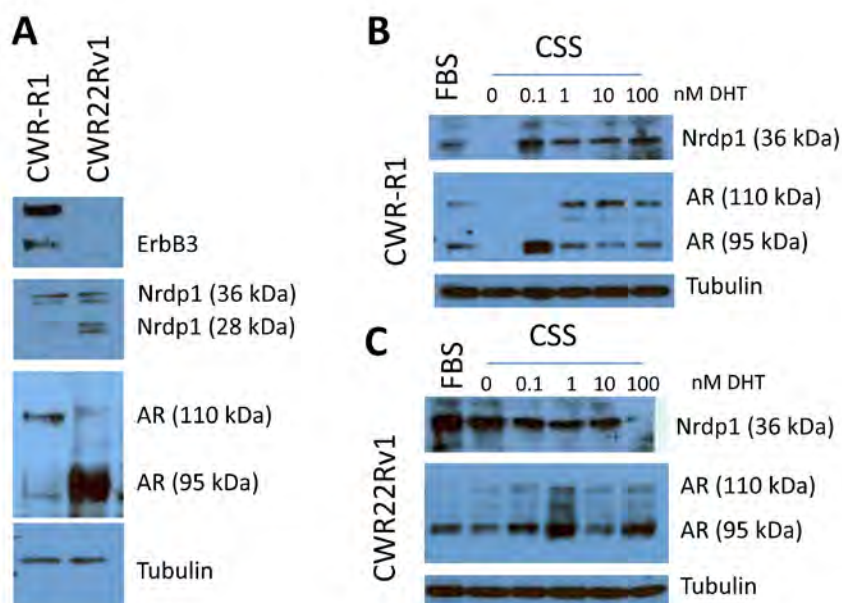


Figure 3. Nrdp1 regulation by AR in Rv1 and R1 cells. (A) AR regulates AR and ErbB3 in R1 but not Rv1 cells. R1 and Rv1 cells were cultured in FBS. Cell lysates were immunoblotted with anti-ErbB3, anti-Nrdp1, anti-AR, and anti-tubulin antibodies. (B) Nrdp1 regulation by AR in R1 cells is androgen dependent. R1 cells were cultured in FBS or CSS medium with increasing concentration of DHT. Cell lysates were immunoblotted with anti-Nrdp1, anti-AR, and anti-tubulin antibodies. As AR expression increases with increasing DHT, so does the expression of Nrdp1. (C) Nrdp1 regulation by AR in Rv1 cells is not androgen dependent. Rv1 cells were cultured in FBS or CSS medium with increasing concentration of DHT. Cell lysates were immunoblotted with anti-Nrdp1, anti-AR, and anti-tubulin antibodies. As AR expression increases with DHT, Nrdp1 expression decreases.

AR binds to ARE3 in the Nrdp1 external promoter in LNCaP, but not C4-2 cells, in an androgen-regulated manner. Chromatin immunoprecipitation (ChIP) on LNCaP cells grown in FBS, CSS, or CSS + DHT showed that the AR bound to ARE3 in these cells only in the presence of androgen (**Figure 4A**). Comparison of LNCaP, LNCaP AI, and C4-2 cells showed that the AR only bound to ARE3 in LNCaP cells (**Figure 4B**).

The transcriptional activity of the AR on ARE3 in LNCaP and C4-2 cells were compared using a normal and a mutant ARE3 luciferase construct (**Figure 4C**), with the addition of DHT, and with bicalutamide, an inhibitor of AR transcriptional activity. Compared with the empty vector, luciferase expression was increased 100 fold by the normal ARE3 and further increased another 300 fold by the addition of DHT, but inhibited by casodex. On the other hand, the mutant ARE3 construct showed no

luciferase activity. This confirmed that the AR bound to ARE3 in LNCaP cells in an androgen-dependent manner. However, when we transfected the two constructs into C4-2, the luciferase expression was no longer regulated by the addition of DHT or bicalutamide, indicating that the AR was no longer androgen dependent (**Figure 4C**).

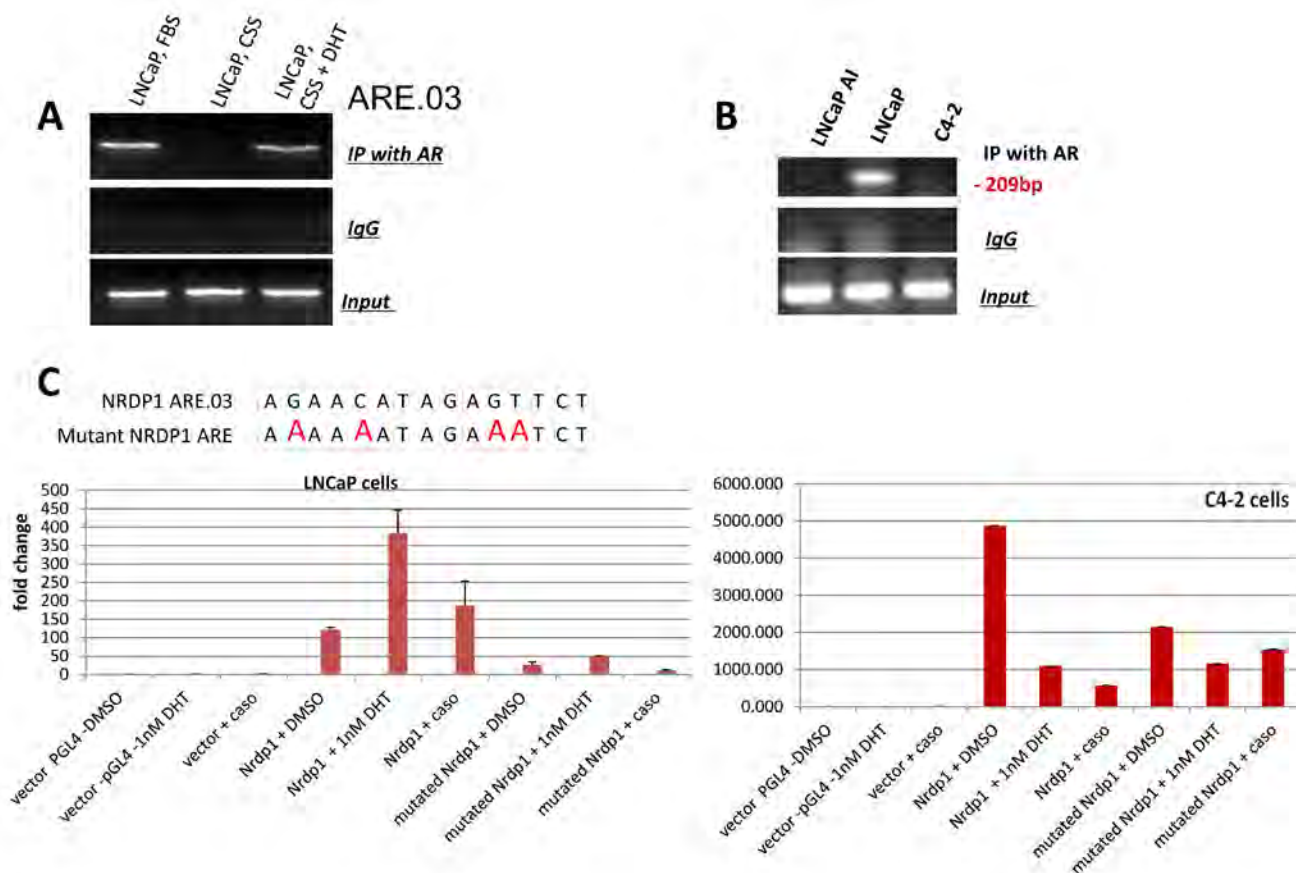


Figure 4. Transcriptional activity of AR on ARE3. (A) ChIP assay of AR binding in LNCaP cells to ARE3. Cells were cultured in FBS medium, CSS medium, or CSS medium with the addition of DHT after the first day. Input is control reactions of genomic DNA prior to immunoprecipitation. Chromatin samples were immunoprecipitated with normal rabbit serum (IgG) or anti-AR antibody and analyzed by PCR with primers flanking the Nrdp1 ARE3 region. (B) AR binds to ARE3 in androgen dependent but not independent cells. ChIP assay of AR binding in LNCaP, LNCaP AI, and C4-2 cells. Cells were cultured in FBS medium. Chromatin samples were immunoprecipitated with normal rabbit serum (IgG) or anti-AR antibody and analyzed by PCR with primers flanking the Nrdp1 ARE3 region. Input is control reactions of genomic DNA prior to immunoprecipitation. (C) Mutation of normal ARE3 to abolish AR binding. Top. Normal Nrdp1 ARE3 and mutant ARE3 were inserted into luciferase constructs to test AR transcriptional activity. Bottom Left. Increased AR transcriptional activity in LNCaP cells on normal ARE3 and normal ARE3 in the presence of DHT compared with control vector and mutant ARE3. Bottom Right. No change in AR transcriptional activity was seen in C4-2 cells on normal ARE3 when compared with control vector and mutant ARE3. Cells were cultured in FBS medium and transfected with the control vector, normal ARE3, or mutant ARE3, and AR transcriptional activity was measured by luciferase assay. Cells were also treated with DMSO, DHT, or casodex.

Expression of a 90 kDa FlnA fragment in C4-2 cells restored androgen responsiveness of the AR and promoted binding to ARE3. We previously showed that LNCaP, but not C4-2 cells, express a 90 kDa cleaved form of the 280 kDa cytoskeletal molecule Filamin A (FlnA) (Colin's reference). Restoration of the 90 kDa fragment in the nuclei of C4-2 cells restored androgen-dependent cell growth while loss of the 90 kDa fragment from LNCaP cells induced castration resistance (Colin's reference). We stably transfected C4-2 cells with FlnA 16-24, C4-2 FlnA 16-24 (**Figure 5A**), and performed ChIP to compare AR binding to ARE3 in LNCaP, LNCaP AI, C4-2, and C4-2 FlnA 16-24. Our results showed that the FlnA 16-24 protein restored AR binding to ARE3 in the C4-2 cells (**Figure 5B**). In addition, luciferase assay was conducted with the normal ARE3 luciferase construct transfected into C4-2 cells with various forms of FlnA including; full-length (280 kDa, which localizes to the cytoplasm), the N-terminal fragment (repeats 1-15, which also localizes to the cytoplasm), and the 90 kDa C-terminal fragment (repeats 16-24, which localizes to the nucleus) (**Figure 5C**). Cells transfected with FlnA 16-24 showed the greatest increase of luciferase activity indicated that the 90 kDa form of FlnA was responsible for restoring AR binding to ARE3 and causing it to act in an androgen dependent manner in these cells.

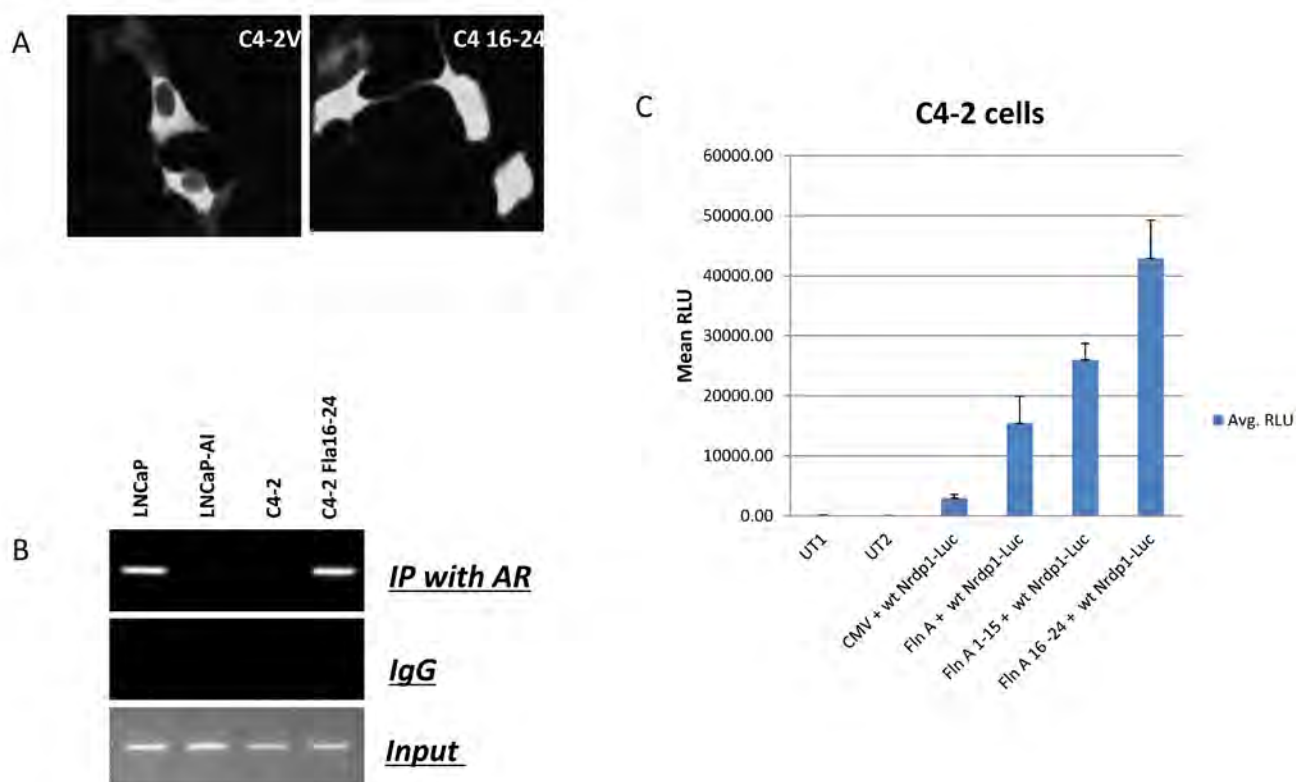


Figure 5. Filamin A restores AR binding to ARE3. (A) Localization of FlnA 16-24 to nucleus. Left. C4-2 cell stained with FlnA conjugated to FITC. Right. Stably transfected C4-2 cells with FlnA 16-24 stained with FlnA conjugated to FITC. **(B)** AR binds to ARE3 in androgen independent cells in the presence of FlnA 16-24. ChIP assay of AR binding in LNCaP, LNCaP AI, C4-2, and C4-2 FlnA 16-24 cells. Cells were cultured in FBS medium. Chromatin samples were immunoprecipitated with normal rabbit serum (IgG) or anti-AR antibody and analyzed by PCR with primers flanking the Nrdp1 ARE3 region. Input is control reactions of genomic DNA prior to immunoprecipitation. **(C)** FlnA restores AR transcriptional activity on Nrdp1 ARE3 in androgen independent cells. Cells were cultured in FBS medium and transfected with full-length FlnA, FlnA repeats 1-15, or FlnA repeats 16-24, and AR transcriptional activity was measured by luciferase assay.

Task 5: In addition, in androgen independent cells we will identify the cause for repression of Nrdp1 expression and investigate whether ErbB3, Akt or its downstream effector FKHRL1 plays a role in this process. (Months 25-36)

Status: Ongoing. These studies will determine whether Nrdp1-regulation by androgens is prevented in androgen-independent prostate cancer cells.

KEY RESEARCH FINDINGS:

In the past two years of this grant award, three papers have already been published with the data generated – one in the first year and two in the second. The two papers from the second year are included in Appendices A and B. A fourth manuscript is now being prepared (based on data collected as Task 4), and demonstrate the following:

- ***ErbB3 stimulates cell growth and is overexpressed in castration resistant prostate cancer.*** Our previous results (reported in the last report) shows that this is because in androgen-dependent cells, ErbB3 expression is suppressed by the androgen receptor (AR), which keeps the cells androgen-regulated. However, in androgen-independent cells, this regulation (of ErbB3 by the AR) is lost, which causes upregulation of ErbB3.
- ***Transcription of Nrdp1, a negative regulator of ErbB3, is androgen-regulated in androgen-dependent LNCaP cells, but not in its androgen-independent subline C4-2 cells.*** Investigation of the mechanism by which the AR regulates ErbB3 in androgen-dependent cells, and also, the mechanism by which this regulation is lost in androgen-independent cells, revealed that the AR regulates the transcription of Nrdp1, a negative regulator of ErbB3 degradation. There are three putative androgen response elements (ARE) in the promoter regions of Nrdp1, two in an internal promoter (ARE1, ARE2) and one in an external promoter just upstream of the transcriptional start site (ARE3). So far, we have been able to confirm AR binding to only ARE3. Significantly, the binding efficiency of AR to ARE3 is stronger in LNCaP cells compared to its androgen-independent sublines.
- ***Differential regulation of Nrdp1 by androgens in different castration resistant prostate cancer cell lines.*** The ability of AR to regulate its downstream target Nrdp1 depends entirely on the ability of the AR to respond to androgens. In cells where the AR is stabilized, androgen withdrawal is not able to cause a decrease in AR levels or transcriptional activity, and hence its downstream targets such as Nrdp1 are unaffected as well. Surprisingly, this is not true for all AR target genes – for example, PSA is equally regulated in all the cell lines tested.
- ***AR binds to ARE3 in the Nrdp1 external promoter in LNCaP, but not C4-2 cells, in an androgen-regulated manner.*** Based on the correlation between AR stability and its transcriptional activity, we see that in cells where the AR is stabilized, Nrdp1 transcription is not affected significantly by androgen addition or withdrawal.
- ***Expression of a 90 kDa FlnA fragment in C4-2 cells restored androgen responsiveness of the AR and promoted binding to ARE3.*** We had earlier shown that androgen responsiveness in prostate cancer cells is regulated by the levels of the structural protein, Filamin A (FlnA) in the nucleus (Wang, et al. 2007). Androgen-dependent cells expressed high levels of this protein in the nucleus while androgen-independent cells did not (Bedolla, et al. 2009). We now show that in cells where FlnA is present in the nucleus, the AR is not stabilized in the absence of androgens and are able to transcriptionally target Nrdp1, whereas in cells where FlnA is absent from the nucleus, AR is stabilized and regulate the transcription of PSA but not Nrdp1. The differential regulation of these two transcriptional targets by the AR is under further investigation.

REPORTABLE OUTCOMES:

1. Publication: Jathal, M.K., Chen, L., Mudryj, M. and **Ghosh, P.M.** Targeting ErbB3: the new RTK(id) on the prostate cancer block. *Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry*, 11(2): 131-149, 2011.
2. Publication: Chen, L., Mooso, B.A., Jathal, M.K., Madhav, A., Johnson, S.D., van Spyk, E., Mikhailova, M., Zierenberg Ripoll, A., Xue, L., Vinall, R. L., deVere White, R.W. and **Ghosh, P.M.** Dual EGFR/HER2 inhibition sensitizes prostate cancer cells to androgen withdrawal by suppressing ErbB3. *Clinical Cancer Research*, 2011, [Epub ahead of print].
3. The latter publication has been featured on MDLinx (<http://www.mdlinx.com/urology/news-article.cfm/3717562>), Silobreaker (http://www.silobreaker.com/dual-egfrher2-inhibition-sensitizes-prostate-cancer-cells-to-androgen-withdrawal-by-suppressing-erbb3-5_2264783756445352038), and in Affective (<http://affective.com/news/dual-egfrher2-inhibition-sensitizes-prostate-cancer-cells-to-androgen-withdrawal-by-suppressing-erbb3>).
4. Qualifying Exam Outcome: Rosalinda Savoy, a graduate student in Dr. Ghosh's lab, is proposing work towards her thesis based on this project.

CONCLUSION: Our data for the first time identifies Nrpd1 as an AR target that is androgen-regulated in castration resistant cells, but not in castration insensitive cells. Our new data shows that in cells where the AR is stabilized, and does not undergo degradation despite androgen withdrawal, it is able to transcribe PSA but not Nrpd1, whereas in cells where the AR is not stabilized, it can transcribe Nrpd1 and thereby regulate ErbB3 levels. Since we also showed earlier that ErbB3 signaling increase cell growth and suppress apoptosis, our results indicate that AR suppression of ErbB3 is a mechanism for keeping cells castration sensitive, whereas when this effect is lost, the cells become castration resistant. Further, we show that Filamin A nuclear localization keeps cells androgen responsive by destabilizing the AR, and maintaining its ability to transcriptionally regulate Nrpd1.

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Wang Y, Kreisberg JI, Bedolla RG, Mikhailova M, deVere White RW & Ghosh PM 2007 A 90 kDa fragment of filamin A promotes Casodex-induced growth inhibition in Casodex-resistant androgen receptor positive C4-2 prostate cancer cells. *Oncogene* **26** 6061-6070.

APPENDICES:

Appendix 1: Jathal, M.K., Chen, L., Mudryj, M. and **Ghosh, P.M.** Targeting ErbB3: the new RTK(id) on the prostate cancer block. *Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry*, 11(2): 131-149, 2011.

Appendix 2: Chen, L., Mooso, B.A., Jathal, M.K., Madhav, A., Johnson, S.D., van Spyk, E., Mikhailova, M., Zierenberg Ripoll, A., Xue, L., Vinall, R. L., deVere White, R.W. and **Ghosh, P.M.** Dual EGFR/HER2 inhibition sensitizes prostate cancer cells to androgen withdrawal by suppressing ErbB3. *Clinical Cancer Research*, 2011, [Epub ahead of print].

SUPPORTING DATA: See above (included in text).

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Targeting ErbB3: the New RTK(id) on the Prostate Cancer Block

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Abstract

Most prostate cancers (PCa) are critically reliant on functional androgen receptor (AR) signaling. At its onset, PCa is androgen-dependent and although temporarily halted by surgically or pharmacologically blocking the AR (androgen ablation), the disease ultimately recurs as an aggressive, fatal castration resistant prostate cancer (CRPC). FDA-approved treatments like docetaxel, a chemotherapeutic agent, and Provenge, a cancer vaccine, extend survival by a scant 3 and 4 months, respectively. It is clear that more effective drugs targeting CRPC are urgently needed. The ErbB family (EGFR/ErbB1, ErbB2/HER2/neu, ErbB3/HER3 and ErbB4/HER4) of receptor tyrosine kinases (RTKs) have long been implicated in PCa initiation and progression, but inhibitors of ErbB1 and ErbB2 (prototypic family members) fared poorly in PCa clinical trials. Recent research suggests that another family member ErbB3 abets emergence of the castration-resistant phenotype. Considerable efforts are being directed towards understanding ErbB3-mediated molecular mechanisms of castration resistance and searching for novel ways of inhibiting ErbB3 activity via rational drug design. Antibody-based therapy that prevents ligand binding to ErbB3 appears promising and fully-humanized antibodies that inhibit ligand-induced phosphorylation of ErbB3 are currently in early development. Small molecule tyrosine kinase inhibitors are also being vigorously pursued, as are siRNA-based approaches and combination treatment strategies- the simultaneous suppression of ErbB3 and its signaling partners or downstream effectors – with the primary purpose of undermining the resiliency of ErbB3-mediated signal transduction. This review summarizes the existing literature and reinforces the importance of ErbB3 as a therapeutic target in the clinical management of prostate cancer.

Keywords

ErbB3; Androgen Receptor; prostate cancer; castration resistance; EGFR; ErbB2; HER2; HER3; lapatinib; erlotinib; trastuzumab

1. INTRODUCTION

The prostate was first described in 1536 but prostate cancer (PCa) was not identified until 1853 [1]. At that time, it was considered a rare disease, likely due to shorter survival, since

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PCa does not affect men until they are older. At the present time, however, it is the most common type of cancer afflicting men in the Western world, with over 2 million currently living with the disease. It is the second leading cause of cancer death in American men after lung cancer. In 2010, at least 217,730 men were diagnosed with prostate cancer and 32,050 were expected to die from the disease (American Cancer Society – Facts and Figures, 2010).

The occurrence and progression of PCa have been linked to the age, race and family history of the patient. 65% of all PCa are diagnosed in men older than 65 [2]. African-American men are three times as likely as Caucasian men are to die from PCa, while Asian-American men are at the lowest risk of developing the disease [2, 3]. Men with a single first-degree relative with a history of prostate cancer are twice as likely to develop PCa, while those with two or more relatives are nearly four times as likely to be diagnosed. The risk increases if the affected family members were diagnosed at a young age and the most susceptible men are those whose family members were diagnosed before age 60.

Patients diagnosed with localized PCa undergo watchful waiting if they are at low-risk or undergo surgery or radiation therapy if they are considered high risk. Prostatectomy, or surgery to remove the prostate, is one of the most common treatments for localized prostate cancer [4]. Radiation therapy is also a common form of treatment for prostate cancer patients. External beam radiotherapy (EBT), co-administered with androgen-ablative treatment, results in improved relapse-free and survival rates and has become the standard-of-care for locally-advanced PCa. In recent years, brachytherapy has also become common in treating subsets of patients with localized PCa [5]. Seeds of radioactive material are implanted in the prostate gland and deliver radiation over a short distance, thereby minimizing damage to normal, non-cancerous tissues.

The majority of patients undergoing treatment for localized prostate cancer respond to these therapies. A small fraction of these patients (15~30%), however, experience tumor recurrence within 5 years following localized treatment, indicating the presence of disseminated disease. These patients are then treated by androgen withdrawal therapy (AW). In the early 1970s, Huggins and Hodges made the seminal observation that androgens played a key role in PCa development and that orchiectomy (removal of the testes) induced cancer regression [6]. Based on their observations, androgen withdrawal continues to be the therapeutic mainstay for disseminated PCa to date; although the majority of patients with metastatic PCa currently are treated with drugs that reduce testicular androgen production, rather than surgery to remove the testes [7].

Androgen withdrawal therapy (AW) is currently the primary, first line, and therapeutic intervention for recurrent prostate cancer [7]. Essentially, AW therapy blocks AR signaling and inhibits the receptor's transcriptional activity. Pharmacological ablation includes gonadotrophin-releasing hormone (GnRH) super-agonists luteinizing-hormone (LH)-releasing hormone (LHRH) analogues, which downregulate the GnRH receptor in pituitary gonadotropes, thus suppressing LH release and inhibiting testicular testosterone secretion [8]. Synthetic GnRH agonists include leuprolide (Lupron), goserelin (Zoladex), buserelin and nafarelin. GnRH antagonists, which inhibit hormone binding to the GnRH receptor, have also been developed as PCa treatments. Several of these antagonists, such as cetrorelix (Cetrotide), abarelix and orgalutran (Ganirelix) are as effective as GnRH agonists in lowering serum testosterone, without causing a testosterone flare associated with GnRH-agonist therapy [9].

The effect of the first line therapy, however, remains in the patient for only about 18-24 months on an average, after which they develop resistance to this therapy. Non-steroidal anti-androgens competitively inhibit the binding of DHT or testosterone to the AR.

Examples within this category are flutamide, nilutamide and bicalutamide (Casodex). This method of treatment constitutes second line therapy and may be used upon failure of first line therapy, either alone, or together with LHRH modulators. Complete androgen blockade (CAB) combines an anti-androgen with a GnRH agonist [10]. This approach benefits about 25-35% of patients initially but does not confer any significant advantage in terms of survival for the majority of PCa sufferers.

Virtually all patients on AW or CAB eventually develop castration-resistant prostate cancer (CRPC) that is refractory to these treatments [7]. The current standard-of-care for CRPC is docetaxel-based chemotherapy, which offers a survival benefit of ~3 months [11], whereas the recently-FDA-approved PCa vaccine Sipuleucel-T (Provenge; Dendreon) extends patients' lifespans by 4.1 months [12]. Hence neither treatment is permanently curative. Patients eventually succumb to the disease [7] and it is clear that more effective therapies are urgently required. A large number of clinical trials have been conducted to identify potential treatments that cure CRPC, but to no avail. Our laboratory has therefore taken the stand that it is more advantageous and feasible to prevent the progression of prostate cancer to CRPC than to cure CRPC after it has already developed. In this review, therefore, we will examine known causes for the development of CRPC and methods by which it could be prevented.

2. FACTORS AFFECTING THE DEVELOPMENT OF CASTRATION RESISTANCE

2.1. Cell Proliferation and Apoptosis in CRPC

In the normal prostate of a mature male, the rate of cellular proliferation (1–2% rate of growth) is balanced by the rate of apoptosis (1–2% per day). This is dependent upon an adequate supply of androgens which ensure that neither involution nor overgrowth of the glands occurs. In contrast, the cancerous prostate suffers from rampant cell growth and/or decreased apoptosis [13, 14]. As described above, PCa cells are initially dependent upon androgens for their sustenance and AW results in tumor regression. It was initially assumed that AW resulted in the apoptotic death of the majority of PCa cells, and that the few that remained were resistant and eventually returned as castration resistant tumors. The number of studies determining proliferation or apoptotic indices in human patients following AW treatment is limited since the majority of patients undergo prostatectomy prior to start of treatment. However in a few reported studies, the results differed widely. Some groups reported increased levels of apoptosis 3 months after AW [15-17], but other investigators found no increase in apoptotic indices in the majority of patients either shortly [18] or 3 months after AW [19]. The authors of the latter study observed that androgen-deprivation was not associated with degeneration or necrosis of neoplastic glands and surmised that AW 'may be related more to suppression of tumor growth than to obliteration of tumor cells'. A similar concept had been put forth earlier [13], that both androgen-dependent and castration resistant human PCa tumors and cells altered their kinetic parameters (i.e., cell cycling status), rendering androgen ablative drugs utterly useless.

Attempts to test this hypothesis in animal models of prostate cancer have also yielded differing results. In the PC-82 and LuCaP xenograft models, increased apoptotic indices were observed following AW [20, 21], whereas in the Dunning R3327PAP rat model tumor growth and mitotic indices were reduced soon after AW but there were no signs of increased apoptosis and tumor cell numbers remained fairly constant throughout the study period [22, 23]. Earlier studies had determined that >80% of non-malignant rat ventral prostatic cells (taken from Sprague-Dawley or Copenhagen males) were lost within 10 days of castration [14, 24], and thus suggested that normal prostatic epithelial cell proliferation and death were differently controlled post-castration when compared to that in prostate tumors. Another

study demonstrated that AW in mice bearing the androgen-dependent CWR22 human prostate tumor xenograft was associated with a decrease in the proliferative index [25], but cellular changes indicative of apoptosis were notably absent. The authors inferred that the tumor cells were growth-arrested in a G0/early G1 state. Later results from the same group corroborated that hypothesis, revealing that the emergence of a castrate-resistant phenotype was associated with release from cell cycle arrest [26].

2.2. AR Signaling and Molecular Mechanisms of Resistance in CRPC

PCa cells rely on the androgen receptor (AR) for proliferation and survival. The AR is activated by ligand-binding and nuclear translocation, dimerization of two AR molecules, and binding to specific androgen-responsive elements (AREs) of androgen-responsive genes and modulating their transcription [27]. The AR is expressed in the majority of prostate tumors, both before and after AW therapy, regardless of their hormone sensitivity [28]. High levels of phosphorylated AR are associated with aggressive clinicopathological features; while increases in AR mRNA and protein levels are necessary and sufficient for progression to CRPC. This in turn is dependent upon a functional AR DNA-binding domain, implying that AR activity and levels are the driving forces for CRPC [27, 28]. The prostate-specific antigen (PSA) gene is an androgen-responsive gene and PSA protein levels are detected in the majority of CRPC, indicating a functional AR-signaling pathway.

Various authors have concluded that there are multiple mechanisms responsible for castration resistance. Overall, there are five principal mechanisms which ultimately increase the AR's cell-growth-promoting functions (Fig. 1) (for a detailed review see [28] and references therein). (i) The androgen receptor is amplified in 25-30% of castrate resistant tumors. Increased AR levels result in increased sensitivity to residual low levels of androgens that are produced by the adrenal gland. (ii) Additionally, in some cases, there is evidence of enhanced rate of T (testosterone)→DHT (dihydrotestosterone) conversion by the enzyme 5 α reductase. (iii) Further, the AR gene itself may be mutated, giving rise to a mutant protein which may be "promiscuous", i.e. can be activated by other circulating steroid hormones (e.g. cortisol) and their metabolic by-products as well as by androgen antagonists like flutamide. These include expression of low molecular weight AR isoforms that are missing the ligand binding domain and are constitutively active allow for AR function in the absence of androgens. (iv) Co-regulator over-expression or co-repressor loss may also facilitate the conversion of anti-androgens into androgen agonists, or allow constitutive activation of the AR, despite the absence of significant levels of androgens in circulation. (v) Constitutive activation of the AR may also result from phosphorylation of the AR by various effectors which allow a configuration change in the AR, resulting in its enhanced transcriptional activity and transcription of target genes in CRPC cells at altered rates compared to castration sensitive cells. Further, altered co-repressor expression and binding and/or AR phosphorylation, also allows altered binding patterns of the AR in CRPC cells compared to its binding in castration sensitive cells [28].

It is of interest to note that most castrate resistant PCa cells, nevertheless, are still androgen sensitive. Although these cells would not cease growth when treated with anti-androgens, they would proliferate at an enhanced rate when challenged by additional doses of androgens [29]. The expression of the AR, is also responsible for cell survival, and in multiple cases, it has been shown that loss of AR expression results in cell death, even in CRPC cells [30-32]. It is likely; therefore, that ligand-dependent AR transcriptional activity is mainly responsible for regulating cell cycle proliferation, while ligand-independent AR activity may additionally regulate cell survival. Hence, androgen withdrawal may result in cell cycle arrest but even in the absence of ligands, the AR may be activated by mechanisms that are independent of ligand binding, which keeps the cells alive. When alternate pathways

that regulate cell cycle progression are activated in CRPC cells, this may result in a release from growth arrest and re-growth of the tumor.

2.3. Activation of Cell Signaling Pathways that Bypass AR Function in CRPC Cells

Studies from different laboratories indicate the existence of alternate pathways in CRPC cells that obviate the need for the AR in regulating the cell cycle pathways. Thus, the AR may be active and functional but cell survival may be regulated by parallel proliferation pathways, mediated, for example, by the serine/threonine kinase Akt [33]. Alternately the growth of the tumor may be facilitated by cancer stem or progenitor cells which do not express the AR but are selected by androgen-ablation therapy as the primary tumor cell type [34]. Alternately, the AR may be activated by a multitude of pathways that confer to it ligand-independent activation resulting in an ability to regulate cell survival, even in the absence of ligands. One of the major causes of re-activation of the cancer promoting pathways in cells that have undergone AW therapy is the phosphatidylinositol 3-kinase (PI3K) pathway. This pathway triggers a number of downstream targets such as Akt (reviewed by us earlier [35, 36]), which promotes cell survival pathways. The stimulation of these pathways prevents cell death during AW treatment [33, 37]. Since receptor tyrosine kinases (RTKs) of the ErbB family are known to turn on the PI3K pathway and regulate AR transcriptional activity in a ligand-independent manner, we will review in the following pages how ErbB receptors, regulate the progression to CRPC.

3. OVERVIEW OF ErbB RECEPTORS; STRUCTURE AND RECEPTOR ACTIVATION

The ErbB family consists of four closely related type 1 transmembrane tyrosine kinase receptors: the epidermal growth factor receptor (EGFR/HER1/ErbB1), ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). Signaling by the ErbB family regulates many cellular activities important for cell survival and function including cell division, migration, adhesion, differentiation and apoptosis. EGFR and ErbB2 have been described in many excellent reviews [38, 39] and hence will be described here only briefly.

3.1. ErbB Receptors are Activated by Ligand Binding, Dimerization and Phosphorylation

The ErbB receptors are activated by mesenchymal ligands – including heregulins (HRG, human) and neuregulins (NRG, esp. mice) and other epidermal growth factor (EGF)-like ligands [40] (Fig. 2). The 4 ErbBs share an overall structure of two cysteine-rich domains in their extracellular region and an intracellular kinase domain, flanked by a carboxy-terminal tail with tyrosine autophosphorylation sites (Fig. 3). Although they have essentially the same domain structure, the functional activity of each varies. ErbB-1, -2 and -4 have active tyrosine kinase domains and ErbB-1, -3 and -4 possess known ligands. ErbB-2 has no known ligand but is constitutively available for dimerization [40]. ErbB-3 can bind several growth factors but until recently was thought to lack intrinsic tyrosine kinase ability (being devoid of the requisite ATP-binding amino acid residues). Recent work has disproved this notion and will be discussed later in this article.

Receptor homo- or hetero-dimerization is imperative for ErbB function and signaling activity. ErbB receptors normally exist as inactive monomers with the homodimerization domains folded to prevent dimerization. Binding of a specific ligand induces a conformational change in the ErbB monomer and readies it for dimerization with a second, active ErbB monomer [40, 41]. The exception may be ErbB2, which is thought to be constitutively activated and readied for heterodimerization. Several different homodimer and heterodimer pairings are possible between the four receptors, with homodimers only weakly perpetuating signals compared to heterodimers (Fig. 2). This ligand-induced dimerization

activates the intrinsic receptor tyrosine kinase activity and leads to trans-autophosphorylation of the monomeric partners [42]. Adapter proteins are recruited to these newly phosphorylated docking sites and a signaling cascade is initiated. It is important to note that ErbB2 and ErbB3 must heterodimerize with the other ErbBs if they are to transmit signals. ErbB2-containing heterodimers are the most potent complexes and the ErbB2-ErbB3 heterodimer is the most mitogenic and transforming of them all.

3.2. ErbB Function in Normal Tissue and in Tumorigenesis

The ErbB kinases are essential for development and tissue maintenance. Although these studies were conducted mostly in EGFR and ErbB2, it gives a broad overview of the functions of ErbB kinases in general. ErbB1 knockout mice die soon after birth, suffering defects in a large number of organs including skin, lung, the GI tract and the brain (reviewed in [43]). Basically, there is immature development in several epithelial organs. In normal mice, the ErbB2/ErbB4 heterodimer acts principally in the heart, whereas ErbB2/ErbB3 function is required for the development of the peripheral nervous system [43]. ErbB2 or ErbB3 knockout mice experience hypoplasia of the sympathetic ganglion chain, loss of cranial sensory ganglia and defective Schwann cell development, due to a loss of migratory ability of cells arising from the neural crest [44]. To circumvent the early lethality of ErbB2 knockout mice, conditional ErbB2 knockout mice have also been developed [45, 46]. Conditional knockdown of ErbB2 in various stages in the life of these mice demonstrated that lack of ErbB2 caused a development of cardiomyopathy, a lack of muscle spindles, defects in muscle regeneration, in effective neuromuscular synapses, abnormally thin myelin sheaths, movement abnormalities and a loss of motoneurons (reviewed in [43]). In the development of the mammary gland, the importance of ErbB1 in ductal growth and the contribution of ErbB2 and ErbB4 for lobulo-aveolar development and lactation has been demonstrated (reviewed in [47]). Based on these reports, it is fairly obvious that ErbB1 has major roles in epithelial cell development whereas ErbB2 plays an important role in cell migration and movement. While these receptors are essential in development, their malfunction later on in life may result in cancer development as well.

In the adult tissue, these receptors and their ligands are still present, but their function may be mainly to maintain the homeostasis of the organ. In cancer, on the other hand, the receptors are inappropriately activated resulting in increased proliferation, decreased survival and increased motility. Based on the existing literature to date, there are three main causes for the role of the ErbB receptors in tumorigenesis: (i) Increased receptor expression and/or gene amplification, (ii) increased ligand expression and (iii) activating mutation of the receptor. Increased expression of ErbB2 has been found to be a common cause for breast cancer [48]. ErbB2 overexpression in breast cancer is associated with poor prognosis, and resistance to hormonal therapy. ErbB2 overexpression has also been associated with metastasis in patients with breast and prostate cancer, especially to the bone [49]. On the other hand, the majority of tumors studied, not only those that are hormonally related, but also other solid tumors, do not exhibit any mutations in ErbB2, or for that matter, in ErbB3 or ErbB4. ErbB3 and ErbB4, when abnormally activated, is more likely to be due to increased availability of their ligands. The same is also true for ErbB1. In the normal prostate, the ligands for these receptors are produced in the stromal tissue, with receptor being expressed in the epithelial cells. In tumors, the epithelial cells themselves may start to produce the ligands, thereby maintaining the receptors in a constant state of activation. ErbB1 receptors, at least in some tumors, especially lung and head and neck, are also prone to mutations that keep these receptors in a constant state of activation [50, 51]. Comparison of the functions of EGFR and ErbB2 in normal development and in cancer indicates that these receptors continue to perform in cancer the tasks that they conducted in development,

which is tissue generation and cell migration, expect that now these tasks are conducted to the detriment of the patient.

In prostate cancer, mutations of any of the erbB receptors have not been seen; however, a large number of studies indicate that EGFR (ErbB1) and ErbB2 (HER2) interact with the AR in the absence of AR ligand binding and stimulate cell survival. The AR was found to both regulate [52] and be regulated by ErbB1 and ErbB2 [53] in castration sensitive, but not in CRPC, human cell lines. In particular, AR expression was suppressed by the activation of ErbB1 [53]; while ectopic expression of ErbB2 was shown to stimulate ligand independent activation of the AR [54]. ErbB2 overexpression in an androgen dependent prostate cancer cell line enhanced AR activity and hormone-independent cell growth [55], whereas small interfering RNA (siRNA)–mediated ErbB2 knockdown impaired prostate cancer cell growth and AR activity [56]. Nevertheless, a large number of ErbB1 and ErbB2 inhibitors were identified which inhibited cell proliferation and survival and also prevented AR transcriptional activity (discussed below). Based on these reports, as well as the fact that ErbB2 regulated PI3K/Akt activation, which made them successful targets of therapy in a number of other solid cancers, ErbB1 and ErbB2 inhibitors were assumed to be the panacea that would kill prostate cancer cells, and prevent castrate resistant prostate cancer.

3.3. ErbB1 (EGFR) and ErbB2 (HER2) Inhibitors in Cancer Therapy

The ErbB family is an established therapeutic target for many human cancers. Anti-ErbB drugs include monoclonal antibodies (MAbs) that target the extracellular regions of the receptor (for example, Trastuzumab, which targets ErbB2), as well as small-molecule tyrosine kinase inhibitors (TKIs) that prevent signal transduction through the receptor's tyrosine kinase domain (for example, erlotinib, which targets ErbB1) (reviewed in [38]). ErbB1 and ErbB2 have been the major recipients of attention with much less consideration given to ErbB3 as a consequence of its impaired kinase activity and previously perceived subservient status compared to ErbB2, which was considered to be the “master positive regulator of the ErbB network” [57]. The anti-ErbB2 monoclonal antibody Trastuzumab was the first inhibitor of the ErbB family to be approved by the US Food and Drug Administration (FDA) in 1998 for the treatment of HER-2 positive breast cancer. Today it is in regular clinical use for the treatment of breast cancer alongside hormone-based therapy. The monoclonal antibody Cetuximab and the small molecule TKIs Gefitinib and Erlotinib target ErbB1 in several types of epithelial cancers and have also received regulatory approval – cetuximab (Erbix) for metastatic colorectal cancer and squamous cell carcinoma of the head and neck, erlotinib (Tarceva) for metastatic pancreatic cancer and non-small-cell lung cancer (NSCLC) and gefitinib (Iressa) for advanced NSCLC [38]. A second-generation, irreversible, pan-ErbB inhibitor presently undergoing clinical trials in patients with advanced lung cancer is PF00299804 [58, 59]. This molecule is a potent inhibitor of ErbB1-activating mutations as well as the ErbB1 T790M resistance mutation both *in vitro* and *in vivo*. The drug also effectively inhibits wild-type ErbB2 and insertion ErbB2 mutations which are observed in the 20-30% of lung cancers that fail gefitinib or erlotinib therapy [58].

3.4. The Failure of ErbB1 and ErbB2 Inhibitors in Prostate Cancer

PCa cells express ErbB1, ErbB2, and ErbB3 receptors [60] so Trastuzumab, Gefitinib and Erlotinib were tested for single-agent therapeutic efficacy in clinical trials in patients with CRPC. No agent, however, displayed any meaningful activity in Phase II trials of men with PCa [61-65]. Preclinical studies had also used Pertuzumab (2C4) - a monoclonal antibody directed against ErbB2 but differed from Trastuzumab in that it prevented ErbB2 heterodimerization with other ErbB family members rather than obstructing ErbB2's ligand-binding domain [38]. Pertuzumab was used to inhibit the growth of CRPC xenografts, while

Trastuzumab used in the same study showed minimal effectiveness in preventing CRPC xenograft growth [66].

In sharp contrast to the preclinical studies, phase II trials of Pertuzumab in patients with CRPC were wholly unsatisfactory - no patient achieved the primary endpoint of >50% decline in PSA [67]. The dual kinase inhibitor lapatinib fared somewhat better in phase II single-agent clinical trials, being fairly well-tolerated and resulting in stable disease for 12 weeks but evidencing no PSA responses [68]. These results challenged the significance of the ErbB1/ErbB2 axis in PCa.

3.5. ErbB3 Activation May Prevent ErbB1 and ErbB2 Inhibitors in PCa

It had been known that while ErbB kinase signals were required for optimal AR function at low levels of androgen, this signaling was mediated not by ErbB1 but by the heterodimerization of ErbB2 with ErbB3 [56]. Sergina *et al.* later demonstrated that ErbB3 was upregulated and provided compensatory signaling precisely in response to ErbB1/ErbB2-directed TKI treatment [69]. ErbB3 activity was characterized by increased membrane localization and phosphorylation. Indeed, ErbB3-directed siRNA duly restored the pro-apoptotic effects of TKIs [69]. These reports suggested that the failure of EGFR and ErbB2 inhibitors may be due to the activation of ErbB3 in these tumors.

Primary PCa cells frequently overexpress ErbB3, which is unaccompanied by increases in ErbB1 or ErbB2 protein [70]. In fact, a surge in the levels – and activation – of ErbB3 is seen when relatively small amounts of ErbB2 are present [71]. Recent work by Soler *et al.* demonstrates that ErbB3 is required for and promotes the invasive capacity of prostate epithelial cells [72]. It achieves this objective by ligand-specific transactivation with either ErbB1 or ErbB2. Castration resistant DU-145 PCa cells were reliant upon ErbB3 expression for optimal motility and clonogenicity *in vitro* and tumorigenicity *in vivo* in response to the NRG-1, EGF and fetal bovine serum [72]. Although MCF-7 breast cancer cells appeared to require ErbB3 as part of an autocrine response induced by EGF and FBS, the response of DU-145 prostate cancer cells to these stimuli, while requiring ErbB3, did not appear to involve autocrine stimulation of the receptor. In both cell types, clonogenicity and tumorigenicity were severely compromised after ErbB3 knockdown with siRNA [72].

ErbB3 has six binding sites for the p85 regulatory subunit of PI3K, as well as for activators of the Ras/mitogen activated protein kinase (MAPK) pathway, and ErbB3-mediated signaling may be responsible for oncogenic cell survival and the promotion of CRPC. As described earlier, AW results in cell cycle arrest whereas CRPC occurs because of release from that arrest. Recent work from our lab shows that in both castration sensitive and CRPC human PCa cell lines and xenografts, AW brought about a visible increase in the protein levels of ErbB3 [73]. This in turn augmented AR transcriptional activity and cell proliferation, signaling the reentry of growth-arrested tumor cells into an actively cycling state. Conversely, ErbB3 downregulation via siRNA suppressed cell viability and impeded CRPC growth [73]. These studies reveal the significant cross-talk between ErbB3 and the AR and indicate a mechanism by which cells may develop resistance to ErbB1 or ErbB2 inhibitors.

4. ErbB3 IN PROSTATE CANCER

4.1. Cellular Localization

The high expression of ErbB3 in certain human cancers suggested that it might be involved in tumor development and, if so, could be marked as a therapeutic target. The cancerous prostate, in comparison to its normal counterpart, overexpresses ErbB3 protein (by IHC visualization [73] and microarray analyses [70]), which indicate poor prognosis. A secreted

isoform of ErbB3 – p45 sErbB3 - was found in PCa bone metastases, activated osteoblasts and new bone matrices but not in the epithelial cells of primary PCa [74]. This isoform stimulated the expression of osteonectin from bone cells which in turn enhanced the invasiveness of PCa cells [75]. It may be mentioned that a secreted, truncated form of ErbB3 – p85 sErbB3 - that acts as a negative regulator of ligand-stimulated ErbB-2, -3 and -4, was found to naturally occur in patients with metastatic breast cancer [76], but has not been studied in PCa patients.

Along with its plasma membranous and cytoplasmic locations, ErbB3, which has a nuclear localization sequence (NLS) near its C-terminal, has been observed in the nuclei of PCa tissues and cell lines. In human PCa tissues, nuclear levels of ErbB3 were low or absent in the benign prostate but increased as the cancer progressed to hormone resistance [77]. Surprisingly, in PCa cell lines, the trend was reversed, with nuclear ErbB3 levels being higher in hormone-sensitive rather than in CRPC cases [77]. As a result, the authors of that study initially associated nuclear ErbB3 staining with risk of disease progression, but in later work discovered that low nuclear localization of ErbB3 was a predictor of biochemical recurrence in patients with PCa and positive surgical margins after radical prostatectomy [78]. ErbB3 expression was also upregulated in the nuclei of PCa cells taken from lymph nodes and bone metastases of patients who had undergone AW therapy [79]. In subcutaneous xenograft tumors of MDA-PCa-2b and PC-3 cell lines, ErbB-3 was predominantly in the membrane/cytoplasm; however, it was present in the nuclei of the xenograft tumor cells implanted in the femur. Castration of mice bearing subcutaneous MDA PCa 2b tumors induced a transient nuclear translocation of ErbB-3, with relocalization to the membrane/cytoplasm upon tumor recurrence [79]. Based on these results, the authors speculate that nuclear localization of ErbB-3 may aid prostate cancer cell survival during androgen ablation and progression of prostate cancer in bone. Based on these results, one can conclude that nuclear localization of ErbB3 may reflect a response to cellular stress (in this case the blocking of AR signaling using an anti-androgen), regulation of RNA synthesis during growth arrest and release from nuclear sequestration in response to proliferation (i.e. when the anti-androgen is removed).

4.2. Ligand-Induced Activation of ErbB3

ErbB3 overexpression does not indicate its activation, since activation requires ligands, dimerization partners, the availability of phosphorylation sites and a variety of intracellular partners to enable signaling. *In vitro* studies suggest that overexpression of a normal receptor leads to transformation only when its appropriate ligand is present; therefore ErbB overexpression has to be accompanied by ligand upregulation (reviewed in [28]). For example, poor prognosis in CRPC directly correlates with overexpressed EGFR, ErbB2, and ErbB3 receptors (at mRNA and/or protein levels) and upregulation of ErbB ligands such as TGF- α , ARG, HB-EGF and EPG. mRNA levels for these ligands were increased 10-100 fold in CRPC as compared to castration sensitive PCa cells [80].

As mentioned earlier, the primary ligands for ErbB3 are members of the NRG family, a large group of isoforms possessing an EGF-like C-terminal and a variable N-terminal region [40]. NRG binding to ErbB3 is followed by ErbB3 heterodimerization, especially with ErbB2. ErbB3/ErbB2 dimerization is favored also by ErbB2 overexpression, which biases heterodimerization towards itself [40]. In the absence of ligand binding, ErbB3 exists in a self-associated, oligomeric, catalytically-inactive state, whereas NRG-bound ErbB3 undergoes a conformational change such that it is stabilized and its extended form exposes the dimerization interface for interaction with ErbB2 [81] (Fig. 4). The extracellular domain of ErbB3 retains NRG-binding ability even at low acidic pH (owing to the absence of a critical, pH-sensitive histidine residue in domain III) indicating a mechanism of survival in the low pH tumor microenvironment [82]. Analysis of PCa cells reveals the existence of a

paracrine loop involving NRG1 and the ErbB3-ErbB2 dimer [60]. The effects of ErbB3 activation by NRG likely depend upon the ratios of NRG isoforms present, their status as secreted (i.e. expressed but unprocessed or sequestered, hence inactive), and the relative amounts of other ErbB receptors.

NRG1 too is overexpressed in PCa and elicits different ErbB3/ErbB2 activation profiles depending upon the hormone-sensitivity of the cells [60]. For example, androgen-dependent LNCaP cells displayed ErbB3/ErbB2 activation, triggering several downstream cascades including PI3K in response to NRG addition [66]. In contrast, CRPC cell lines demonstrated highly variable outcomes – the AR-negative DU145 and PC-3 were unaffected by NRG, CWR22Rv1 demonstrated ErbB3/ErbB2 dimer formation and cell proliferation, and the recurrent PCa cell line CWR-R1 activated an autocrine pathway between NRG and low-level, constitutively-active ErbB3/ErbB2 that led to AR transactivation via the MAPK and PI3K/Akt routes [66, 83]. Significantly, the growth factors EGF and betacellulin, which are not canonical ErbB3 ligands (see Fig. 2), also showed increased binding to ErbB3 co-expressed with ErbB2 but other ErbB family ligands TGF- α , ARG and HB-EGF did not [84]. These reports indicate the ability of the cancerous cell to activate non-specific binding in ErbB3 although the mechanism of action in these cases is not fully known. Ligand-induced activation of ErbB3 is followed by physical association with other ErbB receptors (Fig. 4). It may be noted that ErbB4 expression is lost in most PCa patients, leaving only ErbB1 and ErbB2 available for heterodimerization with ErbB3 [60] (Fig. 5).

4.3. ErbB3 Phosphorylation and Downstream Signaling Partners

ErbB3 heterodimerization is followed by autophosphorylation on tyrosine residues and each receptor thus activates its partner (Fig. 4). Kinases other than ErbB family members can also phosphorylate ErbB3 and notable among these are Src and MET [85, 86]. Both kinases bind to ErbB3, increase its phosphorylation and enhance oncogenic signaling via the ErbB3/ErbB2 heterodimer. Additionally, ErbB3 is activated by the non-receptor Tec family tyrosine kinase Bmx/Etk [87]. In response to ligand stimulation, Bmx/Etk is activated by tyrosine phosphorylation downstream of Src and PI3K in PTEN-deficient PCa cells. Etk downregulation by siRNA markedly decreases PCa cell growth, implying potential validity as a therapeutic target. Other kinase activators of ErbB3 include CDK5 [88], the breast cancer associated BRK/PTK6 [89], transactivation by cellular stress and cytokines like TNF- α and Interferon- α [90, 91]. Janus tyrosine kinases JAK1 and TYK2 have also been implicated as ErbB3 interactors, though neither demonstrated physical association with ErbB3 [92]. The transphosphorylation events resulting from kinase activity create docking sites for adaptor protein binding. These phosphotyrosine binding proteins associate with the tail of each ErbB molecule after engagement into dimeric complexes and determine the specificity and potency of the ensuing intra-cellular signal.

An invariable target of activated ErbB3 heterodimeric complexes is the PI3K/AKT pathway. While ErbB1 and ErbB2 interact with and activate PI3K via adaptor proteins, ErbB3 possesses six binding sites for the p85 regulatory subunit of PI3K, enabling its direct activation [40]. Each of these p85 sites cooperatively contributed to ErbB3 signaling, as was demonstrated by sequential mutation and restoration. Indeed, ErbB3 seems to be the preferred partner when signaling occurs through the PI3K pathway [93]. Activated PI3K phosphorylates AKT which sets in motion the phosphorylation and activation of numerous downstream proteins, resulting in processes that represses apoptosis and promote survival. ErbB3/PI3K/AKT-induced survival and proliferation pathways have been implicated in numerous human cancers and AKT has been singled out for its regulation of CRPC cell proliferation by activating additional signal transduction pathways and stimulating ligand-independent AR activation [29, 35, 36]. Indeed, it has long been known that Akt phosphorylation increases during AW treatment of castration sensitive cells and remains

high in CRPC, but for long, it was not known what factors contributed to this elevation. Our recent work implicates ErbB3 as a possible cause for the increase in Akt phosphorylation since ErbB3 also increased during AW and remained high in CRPC [73]. Therefore the increase in ErbB3 is likely a major cause for the inability of AW to induce cell death.

4.4. Interaction Between ErbB3 and the AR is Mediated by Ebp1

As mentioned in section 2.2, above, the AR is known to remain active in CRPC and continues to regulate signaling pathways that allow them to proliferate and differentiate. There is some evidence suggesting that ErbB3 may be responsible for this ligand-independent AR activation. It was observed that ErbB2/ErbB3 heterodimers, but not ErbB2/ErbB1 units, modulated AR transcriptional activity by stabilizing AR protein and enhancing binding to its cognate AREs [56]. Phosphorylated AR was correlated with activated ErbB3 in animal models and AR-mediated transactivation of reporter genes in human CWR-R1 PCa cells [83].

An intriguing mediator of AR-ErbB3 interaction is the ErbB3 binding protein-1 (Ebp1) [94]. First discovered in a yeast two-hybrid assay, it interacted with the first 15 amino acids of the juxtamembrane domain of unphosphorylated ErbB3, binding directly to ErbB3 only if that RTK was constitutively phosphorylated by PKC [95]. Ebp1 exists as two isoforms that differ in their abilities to bind ErbB3, localize intracellularly and affect cell survival and differentiation [96]. Ebp1 is also recognized as a nucleolar growth regulating factor and an inhibitor of eIF2 α phosphorylation, an initiator of protein translation. Ebp1 is phosphorylated upon NRG stimulation, dissociates itself from ErbB3 and travels to the nucleus. There it interacts directly with the cell cycle regulator pRB, inhibiting transcription of E2F regulated genes by recruiting, among other factors, SIN3A and histone deacetylase (reviewed in [97]).

Ebp1 contains an LXXLL motif that allows it to interact with the AR. It is an AR corepressor which inhibits transcription from AR-responsive gene promoters, including transcription of the AR itself [98, 99]. Ebp1 mRNA and protein levels, therefore, decrease in PCa versus normal prostate tissue [100]. *In vitro* and *in vivo* data demonstrated that Ebp1 overexpression resulted in reduced incidence of LNCaP tumors and slower growth of remaining tumors while siRNA-mediated Ebp1 downregulation in LNCaP cells activated the AR despite absence of androgen [101]. Combined Ebp1 upregulation and cyclin D1 downregulation (Ebp1⁺/D1⁻) predicted PSA relapse, establishing Ebp1's correlation to PCa progression [102].

4.5. Regulation of ErbB3 Levels by the AR is Mediated by Nrdp1

Early work on the regulation of ErbB3 degradation by Nrdp1 was conducted in mammary tumor models and has only recently been applied to PCa. The proteasomal degradation of ErbB3 is regulated by the RING finger E3 ubiquitin ligase Nrdp1 (neuregulin receptor degradation protein 1), also known as RNF41 or FLRF. Like Ebp1, described above, Nrdp1 too was discovered as an ErbB3-interacting protein by yeast two-hybrid analyses and stimulated ErbB3 ubiquitination and degradation in a ligand-independent manner [103]. Thus it regulated the RTK's steady-state levels. Corepressor experiments indicated that Nrdp1 specifically bound to ErbB3 and ErbB4 but not to ErbB1 or ErbB2. The C-terminal domain (CTD) of Nrdp1 directly binds to ErbB3's cytoplasmic tail while the N-terminal RING finger domain is responsible for ErbB3 ubiquitination and turnover. Nrdp1 is itself highly labile, undergoing self-ubiquitination and proteasomal degradation via the deubiquitinating enzyme USP8 [104]. Both proteins – Nrdp1 and USP8 - thus contribute to the efficiency of ErbB3 downregulation by steering it away from the recycling pathway and towards the degradation route. Proteins that target receptors towards ligand-independent

degradation potentially play a significant role in stifling tumor growth properties by suppressing receptor levels. In a transgenic murine model of ErbB2-induced mammary carcinogenesis, the ErbB2 transgene product is highly expressed in tumors but is scarcely detected in non-tumor tissue [105]. Similarly, ErbB3 protein is overexpressed only in tumors and not in uninvolved mammary tissues in these animals. This is not attributed to differences in transcript levels [105]. The same group reported the interesting observation that Nrdp1 protein was present in healthy mammary tissue from the ErbB2-transgenic mice but was completely lost in tumors [105], suggesting that Nrdp1 played the role of tumor suppressant by keeping ErbB3 levels – and signaling - in check.

Little however is known about the expression and function of Nrdp1 in PCa. Recent work from our lab has offered novel insight into one potential mechanism of Nrdp1-mediated CRPC development. We show that ErbB3 protein is negatively regulated by the AR in androgen dependent cells, but not in CRPC cells [73]. AW caused a sharp drop in AR protein levels and transcriptional activity, resulting in the growth arrest of castration sensitive cells. A simultaneous increase in ErbB3 levels was observed in the castration sensitive cells, persisting even after the cessation of AW treatment, which likely drove, at least partly, the eventual growth of the CRPC cells. Continued probe of the AR-ErbB3 relationship uncovered the involvement of Nrdp1, which was found to be under the positive transcriptional control of the AR in castration sensitive cells, and AR-mediated Nrdp1 expression resulted in the ubiquitination and degradation of ErbB3 in these cells. Significantly, CRPC cells, unlike castration sensitive ones, appeared to experience a proliferative advantage because the AR was no longer able to direct the transcription of Nrdp1 in CRPC. The differential regulation of ErbB receptors by the AR in castration sensitive, but not in CRPC cells have also been reported for EGFR and ErbB2 by two separate groups who demonstrated that the AR regulated and was regulated by ErbB1 and ErbB2 in castration sensitive, but not in CRPC, human cell lines [52, 53]. Steroid receptor control of the ErbB receptors likely indicates a mechanism by which the AR suppressed cell growth regulated by the ErbB receptors in castration sensitive cells, and loss of this control with PCa progression may be an important aspect of why and how castration resistance develops.

5. ErbB3 AND TKI RESISTANCE

It is apparent from the above discussion that ErbB3 is intimately involved in the transformative pathways that drive PCa from a castration sensitive to a castration resistant phenotype. Several experimental approaches are being developed using ErbB3 as a therapeutic target. Strategies to target this RTK can broadly be divided into two categories – targeting only the ErbB3 receptor or preventing the formation of ErbB2/ErbB3 oncogenic unit (see below). Among the classes of agents being developed, small molecule tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (MAbs) have gone the farthest. The majority of small molecule TKIs interferes with ATP binding within the receptor's catalytic domain and obstructs trans-autophosphorylation whereas MAbs are raised such that they target the receptor's extracellular region and limit ligand binding. The exception is Pertuzumab which was developed to prevent the dimerization of ErbB2 with ErbB3 (discussed earlier). The end result is that ErbB signaling is inhibited. While we describe a myriad of methods, we note that not all of them have been applied specifically to a PCa model.

The principal signaling function of ErbB3 in cancers was thought to be its role as a binding partner of ErbB1 or ErbB2 and a scaffold for the recruitment of cytosolic signaling proteins. Targeting scaffold functions is difficult for currently available pharmaceutical technologies, and for a long time, ErbB3 lacked a specific inhibitor, particularly since ErbB3 was thought

to lack kinase activity [106]. However, recent data from Shi *et al.* provide surprising evidence of ErbB3's ability to bind to ATP and promote autophosphorylation of the receptor's intracellular domain when clustered at a membrane surface [107]. While ErbB3's tyrosine kinase activity was ~1000-fold lower than that of ErbB1, this small amount of activity was clearly sufficient for the initial autophosphorylation steps. Full kinase activation – or activity that is 150-1000-fold greater – is required only for the receptor to phosphorylate downstream signaling or docking molecules [107]. The weakly-catalytic ErbB3 thus efficiently phosphorylates ErbB2 whose vastly superior kinase activity then takes up the task of phosphorylating downstream substrates, propagating the pro-survival signal in a rapid and robust manner. ErbB3 autophosphorylation *in vitro* is uninhibited by single inhibitors of ErbB1 or ErbB2, displaying the probable culpability of residual ErbB3 kinase signaling in promoting TKI resistance [107].

Despite the current finding of weak intrinsic kinase function in ErbB3, it is still difficult to target the function of this RTK because the overall role of the kinase function is relatively low-grade compared to its function in heterodimer formation and in scaffolding. To overcome this drawback, and yet recognizing the importance of ErbB3 in different cancers, pharmaceutical companies and other investigators have taken innovative approaches to inhibit this RTK. Below, we will discuss possible methods of inhibiting ErbB3 signaling, some intentional and some fortuitous (see Table 1).

5.1. Monoclonal Humanized Anti-ErbB3 Antibodies

ErbB3's signaling functions depend upon ligand binding to its extracellular domain and inhibitors are generated to disrupt this interaction. A recently-characterized, ErbB3-specific humanized antibody MM-121 blocked ligand-dependent ErbB3 activation induced by the ErbB1, ErbB2 or MET receptors [108]. This MAb was tested in a variety of human cancer cell lines and tumor xenograft models (lung, renal, gastric, breast and ovarian) and worked most efficiently in those cancers that overexpressed the ErbB3-specific ligand heregulin. The aggressive human prostate cancer cell line DU-145 also fell into this category, for it harbors a strongly-activating, ErbB3-heregulin autocrine loop. In contrast, the Ab fared poorly in cells with an amplified ErbB2 gene because their growth was likely driven by ligand-independent and not ligand-dependent mechanisms. MM-121 is currently in clinical development as a therapy against a variety of cancers [108].

Another ErbB3-targeted MAb is AMG-888 (U3-1287, NCT00730470) - *in vitro* studies showed that AMG-888 was able to inhibit the growth of multiple tumor cell lines (breast, lung, colorectal) that were resistant to other ErbB family inhibitors¹. Additionally, AMG-888 demonstrated statistically significant growth inhibition of established xenograft tumors as a single agent and in combination with other ErbB family inhibitors. This fully-humanized MAb is currently in Phase I trials in patients with advanced solid tumors that have become refractory to standard therapy or for which no acceptable treatment currently exists. AMG-888 prevents ligand-induced phosphorylation of ErbB3, ErbB2, and downstream effector molecules including Akt, ERK1 and ERK2. *In vivo* studies show that colony formation in pancreatic cancer cells and tumor growth in pancreatic, non-small cell lung cancer, and colorectal xenograft models are both significantly decreased following treatment with this drug (see also [109]).

¹Freeman, D., S. Ogbagabriel, M. Rothe, R. Radinsky, and M. Treder. Fully human anti-HER3 mAb U3-1287 (AMG 888) demonstrates unique *in vitro* and *in vivo* activities 309 versus other HER family inhibitors in NSCLC models. Proceedings of the 99th Annual Meeting of the American Association for Cancer Research. 2008. San Diego, CA, USA.

5.2. Dual- or Multi-ErbB Inhibitory Approach

It should be clear by now that the ErbB receptors cooperate with each other in driving signal transduction towards malignant transformation. The mutual interactions that exist between these receptors tend to compromise the success of drugs that target individual receptors in cancer treatment. Preclinical studies show that tumor cells can rescue themselves, in more ways than one, from the inhibitory effects of an agent directed toward one ErbB receptor. They may alter their activation ability by relying on the ligand for a different ErbB receptor [110], shifting their signaling profiles such that an untargeted receptor is made to drive cellular growth [69, 111] or co-opting an entirely different RTK into a pro-survival, heterotrimeric supercomplex [112]. In all cases, signaling is but temporarily halted, only to inevitably return stronger than before. On the other hand, both *in vitro* and *in vivo* models have shown that employing a dual- or multi-ErbB inhibitory approach demonstrates greater anti-tumor activity than agents targeting an individual ErbB receptor [113-117]. Strategies involve putting together two types of MABs, combining TKIs with MABs or administering single molecules that inhibit one or more ErbBs simultaneously (discussed later). In the case of ErbB3, MM-121 combined with the anti-ErbB1 MAB cetuximab led to prolonged RTK inhibition in a mouse lung cancer model when compared to MM-121 alone [108]. As an ErbB-targeted approach, the combination of a MAB and TKI uses two agents with different sites of action. For example, trastuzumab plus the dual ErbB1/ErbB2 inhibitor lapatinib given to patients with metastatic breast cancer increased progression-free survival rate [118]. Among the reasons proposed for their therapeutic synergy was the ability of lapatinib (but inability of trastuzumab) to bind to truncated ErbB2 [93], often overexpressed in metastatic breast cancer.

Multi-ErbB inhibitors are being pursued most vigorously and antagonize the actions of ErbB heterodimers or inhibit, at one time, more than one individual ErbB receptor. Implicit in the inhibition of the ErbB1/ErbB2 heterodimer is the notion that ErbB3 too will be deactivated for lack of available ErbB dimerisation partners, especially in diseases like PCa where the fourth member of this family, ErbB4, is lost [60] (Fig. 6). Of note is the fact that the newer pan-ErbB inhibitors also aim at directly disrupting ErbB3 activity.

The first-generation, irreversible, pan-ErbB inhibitor canertinib (CI-1033) inhibited TK activity of all the ErbB family members without affecting other RTKs (PDGFR, FGFR, IGFR) even when administered at high concentrations to a variety of human cancer cell lines, including PCa cell lines [119]. It is interesting to note that canertinib also induced G1 cell cycle arrest and apoptosis in an ErbB-independent manner in cell lines derived from human pre-myelocytes and histiocytic lymphomas [120]. While transcripts for all ErbBs were readily detected in these cell lines, protein expression was absent. This raises the possibility of canertinib exerting an off-target effect through an as-yet undetermined molecular mechanism, possibly involving the inhibition of mRNA translation of the ErbB receptors [120]. Canertinib is currently in Phase II clinical trials for the treatment of patients with advanced-stage non-small cell lung cancer (NSCLC) [121].

The pan-TKI MP470 was designed using a structure-based approach and inhibited cell proliferation in human castration resistant and CRPC cell lines [122]. When co-administered with erlotinib in the context of an LNCaP mouse xenograft model, the drugs not only completely abrogated ErbB1, ErbB2 and ErbB3 phosphorylation, but also prevented ErbB3 binding to PI3K and inhibited downstream Akt activity, even in androgen-depleted conditions. The safety and efficacy of the MP470-erlotinib combination is currently being evaluated in Phase 1 clinical trials for refractory solid tumors [122].

One of the most recently-documented pan-ErbB inhibitors is AstraZeneca's AZD8931 [123], shown to have activity as an equipotent TKI against ErbB1, ErbB2 and ErbB3 signaling in a

variety of human head and neck, non-small-cell lung and breast cancer cell lines and murine xenograft models. The drug displayed greater inhibitory activity towards the ErbB3/ErbB2 oncodimer and was expected to be of particular use in solid tumors that did not contain amplified ErbB2 or mutated ErbB1 genes.

Another pan-ErbB inhibitor mentioned above is PF00299804, a potent inhibitor of EGFR-activating mutations as well as the EGFR T790M resistance mutation both *in vitro* and *in vivo* [58]. PF00299804 also inhibits both wild-type and gefitinib-resistant mutated ErbB2 identified in lung cancers [58]. Increased expression of ErbB3 was shown to induce resistance to PF00299804 [124]. This drug is an irreversible inhibitor of ErbB1 [58], which has been shown to inhibit the growth of various cell lines overexpressing ErbB3 [59].

One of the most successful pan-ErbB inhibitors have been lapatinib (GW275016) which has been mentioned throughout in this review. Tyrosine phosphorylation of ErbB2 and ErbB3, AR transactivation, and cell proliferation induced by heregulin were more potently inhibited by lapatinib than the EGFR-specific inhibitor gefitinib [83]. Basal proliferation in the absence of growth factors was also inhibited by lapatinib to a greater extent than gefitinib, suggesting that low level HER2/H ER3 activation perhaps by an autocrine pathway contributes to the proliferation signal [83, 125]. As mentioned earlier, a Phase II multicenter clinical trial to evaluate Lapatinib in early stage, hormonally untreated recurrent or metastatic prostate cancer was unsuccessful [68], but will be discussed further in the section below.

5.3. Effectiveness of Dual ErbB1/ErbB2 Inhibitors in Combination with AW Therapy

As mentioned earlier in this article, activation of the ErbB2/ErbB3 signaling cascade can lead to constitutive, ligand-independent activation of the AR and render PCa cells indifferent to AR inhibition [55, 56]. In fact, activation of the ErbB receptors, leading to stimulation of parallel signaling pathways that bypass the AR and regulate cell signaling and survival independent of the AR, is a major cause of the development of CRPC (see section 2.2). On the other hand, merely inhibiting ErbB2, or dual ErbB1/ErbB2 or even pan-ErbB inhibitors were insufficient to inhibit cell growth completely in patients with CRPC [67], given that this disease is associated with a large number of aberrations, many of which are associated with increased activation of the AR. Therefore, it is more reasonable to utilize the ErbB inhibitors at an earlier stage in order to prevent the progression of the disease. Rather than apply these drugs to patients with CRPC, they may be better used in hormone-sensitive patients when combined with anti-androgens.

Indeed, applying an ErbB inhibitor alongside an AR inhibitor appears to be more efficacious, at least in initial studies. For example, in MDA PCa 2a prostate cancer cells, the AR antagonist hydroxyflutamide proved more efficacious when combined with cetuximab and trastuzumab [126]. Significantly, in androgen-dependent PCa cell lines, co-administration of gefitinib and bicalutamide resulted in concurrent inhibition of AR and ErbB1/ErbB2 pathways, causing a significant delay in the onset of ErbB-driven castration resistance [127]. The same principle has been suggested for PCa patients who have undergone radical prostatectomy and radiation therapy - lapatinib plus an anti-androgen appear to offer a better therapeutic option than lapatinib alone².

The problem with anti-androgens is that the patients acquire resistant to this treatment fairly quickly. Acquisition of resistance employs multiple mechanisms including the failure of the drug to bind to its target. In that case, alternate mechanisms of action to decrease AR

²Chen, Y., G. Wilding, J. Gee, R.P. DiPaola, M. Pins, M.A. Carducci, M.N. Stein, G. Bubley, and G. Liu; A phase II trial of lapatinib (GW572016) in patients with recurrent prostate cancer as evident by a rising PSA. *J Clin Oncol*, **2008**. 26 (15), 5170-5170.

transcriptional activity are needed. Clinical resistance to TKI therapy is also associated with re-activation of PI3K signaling [69]. The combination of anti-ErbB/anti-PI3K therapeutics is effective in animal models and is undergoing extensive clinical testing [128]. There has been emphasis on the use of PI3K inhibitors in tumors that are resistant to the ErbB1 or ErbB2 inhibitors Erlotinib, Lapatinib, and Trastuzumab because the resurgence of PI3K signaling is largely due to the direct activation of upregulated ErbB3 [129-131].

6. CONCLUSIONS AND FUTURE DIRECTIONS

The preponderance of literature leads to the conclusion that CRPC arises because a few (or more) tumor cells survive first line AW therapy and then recur with an altered phenotype that no longer respond to this therapy. Hence, if the existing tumor cells are all eliminated completely, then the chances of the tumor recurring are reduced to a large extent, regardless of whether the tumor arises by alterations in existing tumor cells or whether cancer stem cells give rise to new tumors that are castration resistant. Activation of the PI3K pathway appears to be a major factor in the ability of the cells to survive, whether by apoptosis or by the triggering of autophagy. Therefore, disruption of the cell survival mechanism during AW seems to be a promising method by which CRPC can be prevented to a large extent.

Disruption of the PI3K/Akt pathway directly is of course possible, but Akt is such an important mechanism in the survival of all the cells in the body, that systemic inhibition of Akt phosphorylation is bound to have a tremendous impact on the survival of normal cells as well. Indeed, in Phase II clinical trials, the Akt inhibitor perifosine was shown to cause Grade 1-2 fatigue and gastrointestinal toxicities [132], and Grade 3 dose-limiting toxicities resulting in hyponatremia, arthritis, hyperuricemia, and photophobia [133]. Indeed, since the ErbB receptors are major activators of the PI3K/Akt pathway, it may be advantageous to inhibit the ErbB receptors directly. However, as has been shown above, inhibition of EGFR or ErbB2 individually did not seem to have a significant impact in clinical trials. We also offer proof that the failure of these single EGFR and ErbB2 inhibitors may result from the activation of ErbB3, and that dual inhibition of EGFR and ErbB2 may fare better, especially in patients undergoing AW therapy². This observation is all the more significant because we have shown that AW therapy at the cellular level induces an increase in ErbB3 levels that may contribute to the induction of the CRPC phenotype [73].

Fig. (6) summarizes how the presence of ErbB3 prevents the effect of individual inhibitors of EGFR and ErbB2 on cell survival. Most prostate cancer cells do not express ErbB4 [60, 134], indeed, expression of ErbB4 appeared to disrupt the growth of prostate cancer cells [135, 136]. Therefore, the only possible ErbB dimers in PCa are EGFR homodimers and ErbB1-ErbB2, ErbB2-ErbB3, and ErbB1-ErbB3 heterodimers. Individual inhibition of EGFR using specific and selective inhibitors would disrupt the functioning of EGFR homodimers and ErbB1-ErbB2 and ErbB1-ErbB3 homodimers, but signaling would still continue through the ErbB2-ErbB3 heterodimers. Similarly, individual inhibition of ErbB2 would prevent signaling downstream of ErbB1-ErbB2 and ErbB2-ErbB3 heterodimers but allow signaling downstream of EGFR homodimers and ErbB1-ErbB3 heterodimers. However, dual inhibition of both EGFR and ErbB2 would inhibit all 4 dimers, thereby completely stopping the abnormal activation of downstream targets through the ErbB receptors.

Since it has become clear that ErbB3 occupies a prominent role in regulating cellular processes that promote CRPC future studies that explore in greater detail previously uncharacterized aspects of ErbB3 biology are warranted. What roles do the truncated isoforms of ErbB3 play, given their opposing functions? Recent clinical findings indicate that p45 sErbB3 could be involved in the bone-forming pheno-type typical of bone metastases in PCa

[137]. The novel ErbB3 isoform p85 sErbB3 may be an ideal candidate for cancer drug development, given its effectiveness at blocking HRG-induced cell growth [76]. What is the importance of ErbB3's nuclear and nucleolar localization? Recent work has revealed a vast array of interesting proteins - for example, Ras regulatory molecules and proteins involved in cell motility - that might bind to ErbB3 and promote ErbB3-mediated tumorigenesis [84]. The molecular basis of these interactions, as well as those involving ErbB3 regulation by the non-ErbB tyrosine kinases Src, MET and CDK5 (among others) remain unknown and merit further investigation. The widely-expressed Ebp1 has presented itself as a viable therapeutic target in CRPC and it would be interesting to learn of studies that advanced this premise. However, Fig. (6) also shows the limitations of single therapy using ErbB3 inhibitors. We conclude that ErbB3 inhibitors in combination with other related inhibitors may be of interest in the prevention of prostate cancer progression to CRPC.

ABBREVIATIONS

AR	Androgen Receptor
ARE	Androgen Response Element
ARG	Ampiregulin
AW	Androgen withdrawal
BRK/PTK6	Breast tumour kinase/Tyrosine-protein kinase-6
CAB	Complete androgen blockade
CDK5	Cyclin-dependent kinase-5
CRPC	Castration Resistant Prostate Cancer
CTD	C-terminal domain
DHT	Dihydrotestosterone
Ebp1	ErbB3 binding protein-1
EBT	External beam radiotherapy
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
eIF2α	Eukaryotic translation initiation factor 2
EPG	Epiregulin
ErbB	Erythroblastic Leukemia Viral Oncogene Homolog
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
FGFR	Fibroblast growth factor receptor
FLRF	Fetal Liver Related Factor
GnRH	Gonadotrophin-releasing hormone
HB-EGF	Heparin-Binding Epidermal Growth Factor
HER	Human Epidermal growth factor Receptor
HRG	Heregulins
IGFR	Insulin-like growth factor receptor

IHC	Immunohistochemistry
JAK	Janus kinase (“just another kinase”)
LHRH	Luteinizing-hormone-releasing hormone
MAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MET	MNNG HOS Transforming gene
NLS	Nuclear localization sequence
Nrdp1	Neuregulin receptor degradation protein 1
NRG	Neuregulin
NSCLC	Non-small-cell lung cancer
PCa	Prostate Cancer
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein Kinase C
pRB	Retinoblastoma gene product
PSA	Prostate-specific antigen
PTEN	Phosphatase with tensin homology
RING	Really interesting new gene
RTK	Receptor tyrosine kinase
siRNA	Small Interfering RNA
TGF	Transforming Growth Factor
TK	Tyrosine Kinase
TKI	Tyrosine kinase inhibitor
TNF	Tumor Necrosis Factor
TYK2	Tyrosine kinase-2

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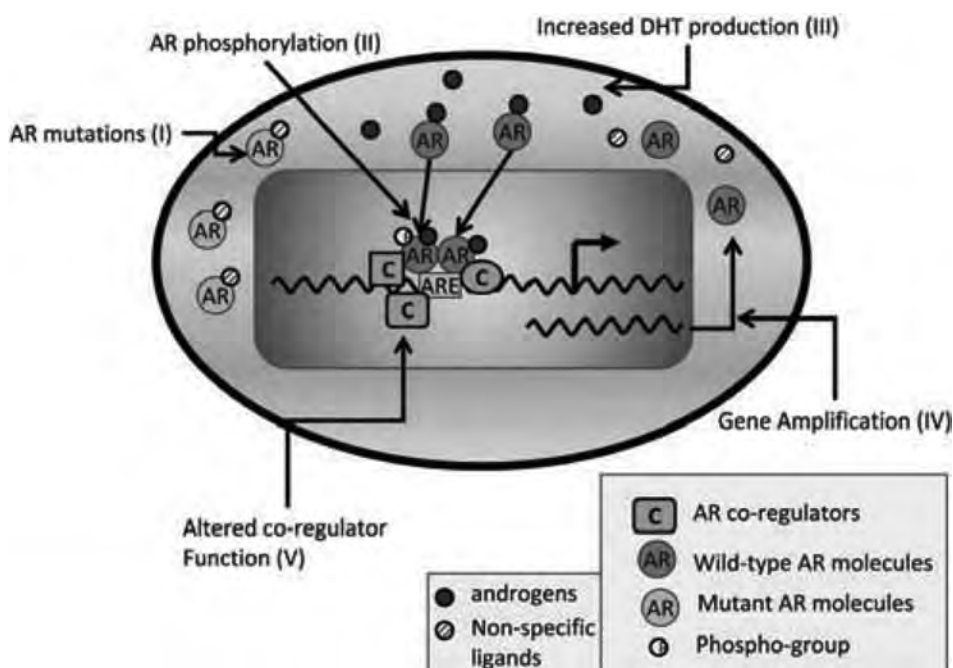


Fig. (1). Molecular mechanisms of castration resistance

Normal prostate and ADPCa cells are dependent on ligand-driven AR activity for their growth and survival. The AR is activated by binding to its ligands, translocating to the nucleus, homodimer formation and binding to specific androgen-responsive elements (AREs) of androgen-responsive genes and modulating their transcription. On the other hand, CRPC cells activate mechanisms that enable their survival in an environment with castrate levels of androgen. These include (i) mutations in the AR, (ii) ligand-independent AR phosphorylation and activation, (iii) increased AR ligand production, (iv) AR gene amplification and (v) altered functions of AR co-regulatory proteins. Different shapes of the co-regulators (C) represent different types of coregulators that bind to the AR.

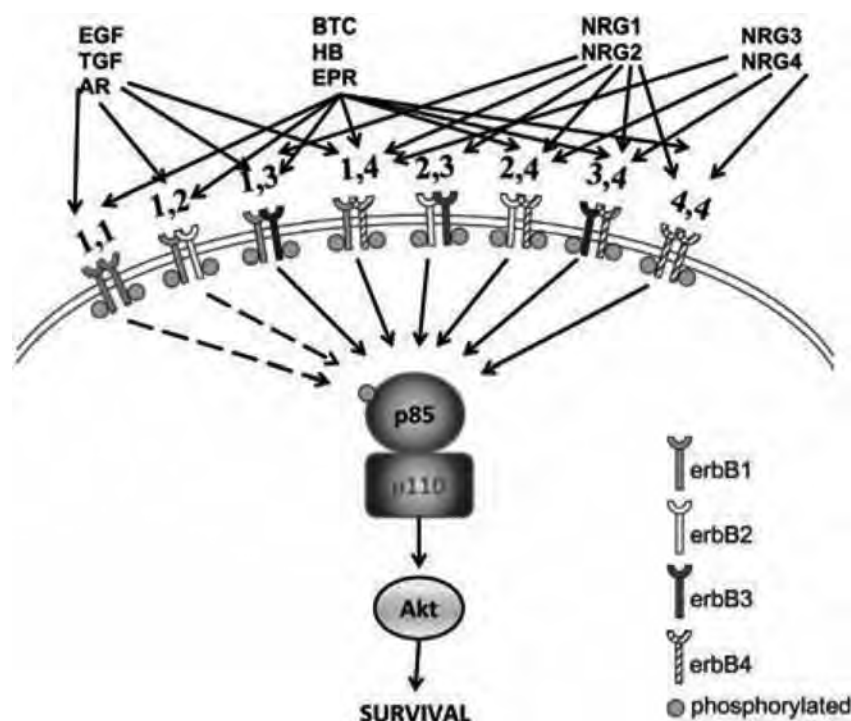


Fig. (2). ErbB family signaling

Three groups of ligands bind to ErbB family receptors. EGF (epidermal growth factor), ARG (amphiregulin) and TGF- α (transforming growth factor alpha) bind to ErbB1; BTC (betacellulin), HB-EGF (heparin-binding EGF-like factor) and EPR (epiregulin) bind to ErbB1 and ErbB4; NRG-1 and NRG-2 (neuregulins 1, 2) bind to ErbB3 and ErbB4; NRG-3 and NRG-4 (neuregulins 3,4) bind only to ErbB4. Possible receptor pairings are shown (note that ErbB3 cannot homodimerize owing to its weak kinase activity and ErbB4 is absent in prostate cancer). ErbB dimers activate pro-survival pathways mediated by Akt (shown here) as well as other pathways not shown. ErbB3 is unique because it binds directly to PI3K which in turn associates directly with and activates Akt, which is directly known to stimulate cell survival.

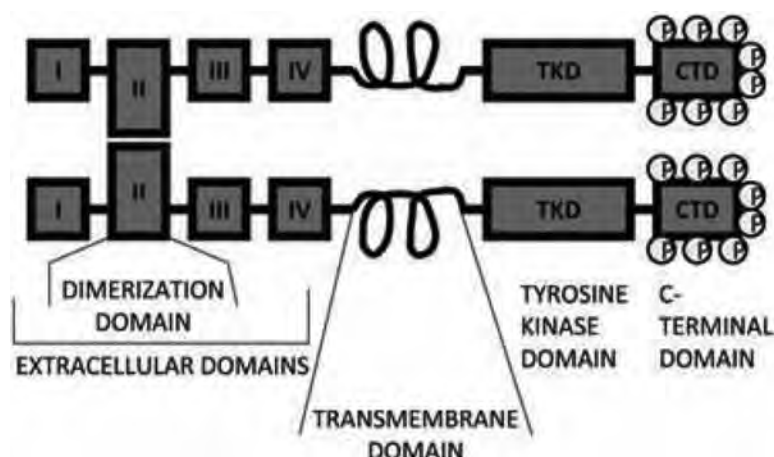


Fig. (3). Schematic of ErbB structure

All members have a large extracellular ligand-binding region (consisting of subdomains I-IV), a single, small intracellular transmembrane-spanning region (which precedes the cytoplasmic tyrosine kinase domain) and a C-terminal tail, which houses the docking (ie phosphorylation) sites for phosphotyrosine-binding effector molecules. Subdomains I and III are leucine-rich repeats that function in ligand binding (also called L1 and L2), whereas subdomains II and IV are laminin-like, cysteine-rich domains (also called CR1 and CR2). The monomeric ErbB receptor is autoinhibited by the interaction of domain II with domain IV. This keeps subdomains I and III apart and prevents ligand binding by disrupting the ligand-binding pocket and burying the dimerization loop of domain II. Ligand binding relieves these inhibitory interactions and encourages dimerization by allowing the loop from domain II of one monomer to access the docking site on domain II of a second, ligand-bound monomer. The receptor dimer is thus stabilized, the kinase domain is activated and specific tyrosine residues within the cytoplasmic tail are phosphorylated. These phosphorylated residues serve as docking sites for a range of proteins and the subsequent activation of intracellular signalling pathways.

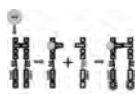


Fig. (4). NRG activation of ErbB3

ErbB3 has a high affinity for NRG and this is greatly increased by dimerization with ERBB2. As with the other ErbBs, in the absence of ligand, a direct intramolecular interaction between domains II and IV keeps ErbB3 in a closed (locked or tethered) conformation that prevents interaction between domains I and III. This conformation disrupts the ligand-binding pocket and buries the dimerization arm of domain II. ErbB2 is inherently unable to dimerize because of a strong interaction between domains I and III which leads to a constitutively extended dimerization arm. ErbB2 is therefore constantly primed for interactions with ligand-bound receptors of the ErbB family. In the presence of NRG, the dimerization loop from domain II of ErbB3 extends to interact intramolecularly with a ligandless, primed ErbB2 monomer to form the oncogenic ErbB2-ErbB3 heterodimer.

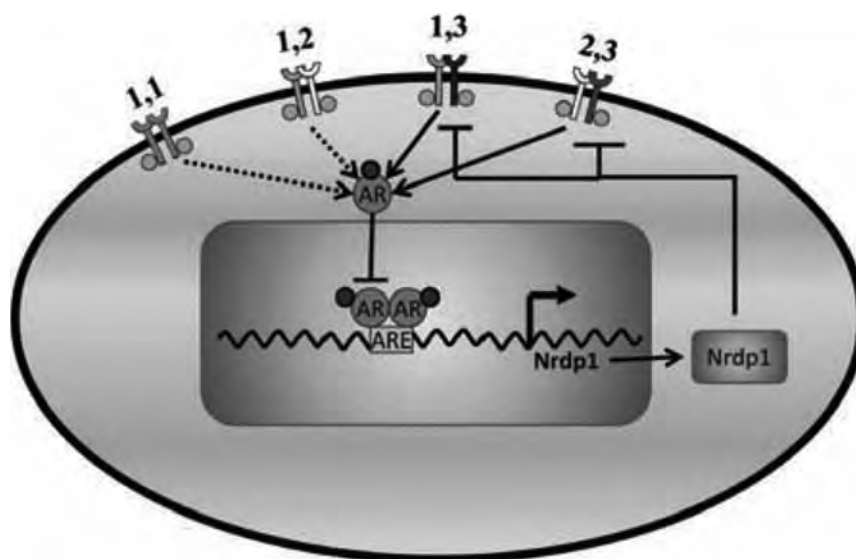


Fig. (5). AR controls ErbB3 levels via transcriptional control of the E3 ubiquitin ligase Nrdp1
 Activated AR enters the nucleus and binds to androgen response elements (ARE) in the Nrdp1 promoter region, initiating transcription of that molecule. Nrdp1 thus produced attaches ubiquitin to ErbB3 and marks it for proteasomal degradation, thereby regulating receptor levels. This regulation occurs in castration-sensitive PCa but is lost en route to castration-resistance. As a result, ErbB3 levels remain sufficiently high and continue to drive tumorigenic growth.

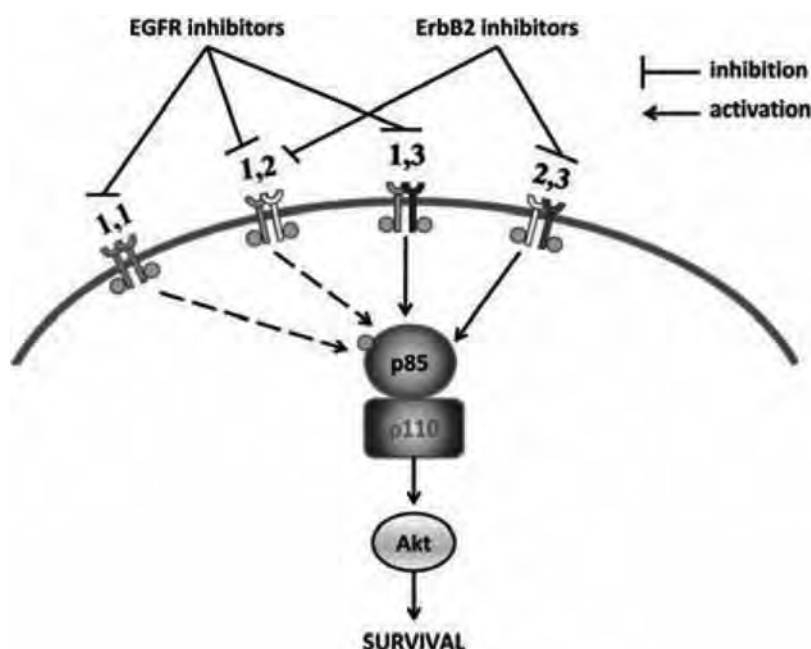


Fig. (6). Inhibition of ErbB3 signaling using a multi-receptor targeting approach

The simultaneous inhibition of ErbB1 and ErbB2 in PCa will leave no dimerization partner for ErbB3 and halt its oncogenic signaling. The only possible ErbB dimers in PCa are EGFR homodimers and ErbB1-ErbB2, ErbB2-ErbB3, and ErbB1-ErbB3 heterodimers (see text). All these dimers would stimulate cell survival, for example, through the PI3K/Akt pathway (shown) as well as by other pathways (not shown). ErbB1 inhibitors would disrupt ErbB homodimers and ErbB1-ErbB2 and ErbB1-ErbB3 heterodimers, but signaling would still continue through the ErbB2-ErbB3 heterodimers. Similarly, ErbB2 inhibitors would prevent signaling downstream of ErbB1-ErbB2 and ErbB2-ErbB3 heterodimers but allow signaling downstream of EGFR homodimers and ErbB1-ErbB3 heterodimers. However, dual inhibition of both EGFR and ErbB2 would inhibit all 4 dimers, thereby eliminating cell survival downstream of the ErbB receptors.

Table 1

List of ErbB Inhibitors Described in this Review

Name of Drug	Class	Target	Current Status
MM-121	Monoclonal humanized ErbB3 antibody	ligand-dependent ErbB3 activation	Phase II for triple-negative breast cancer, Phase I/II for advanced NSCLC, Phase I for gynaecological cancers
AMG-888 (U3-1287)	Monoclonal humanized ErbB3 antibody	ligand-induced phosphorylation of ErbB3	Phase I for advanced NSCLC and advanced solid tumors
Canertinib (CI-1033)	irreversible pan-ErbB TKI	ErbB tyrosine kinase domain	Phase II for refractory metastatic breast cancer and advanced NSCLC
MP-470	pan-ErbB inhibitor (ErbB1, 2, 3)	ErbB phosphorylation	Phase I for advanced solid tumors
AZD8931	reversible pan-ErbB inhibitor (ErbB1, 2, 3)	ErbB phosphorylation	Phase I for advanced solid tumors, Phase II for breast cancer
Trastuzumab (Herceptin)	monoclonal humanized ErbB2/ ErbB3 antibody	ligand-dependent ErbB3 activation (prevents ErbB3/ErbB2 dimerisation)	FDA-approved for metastatic breast cancer
Erlotinib (Tarceva)	reversible ErbB 1 TKI	Prevents ATP binding to ErbB 1 TK domain	FDA-approved for metastatic NSCLC
Cetuximab (Erbix)	monoclonal humanized ErbB1 antibody	ligand-dependent ErbB1 activation (prevents ErbB3/ErbB1 dimerisation)	FDA-approved for irinotecan-refractory colon cancer and advanced head-and-neck cancers
Lapatinib (Tykerb)	Dual TKI inhibitor (ErbB1, 2)	ErbB tyrosine kinase domain	FDA-approved for breast cancer (triple-positive)
PF00299804	pan-ErbB inhibitor (ErbB1, 2, 4)	ErbB tyrosine kinase domain	Phase II for advanced NSCLC
Pertuzumab (Omnitarg/2C-4)	monoclonal humanized ErbB2 antibody	ligand-dependent ErbB2 activation (prevents ErbB3/ErbB2 dimerisation)	Phase II for advanced solid tumors
Gefitinib (Iressa)	reversible ErbB1 TKI	Prevents ATP binding to ErbB1 TK domain	FDA-approved for metastatic NSCLC

Clinical Cancer Research



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Dual EGFR/HER2 inhibition sensitizes prostate cancer cells to androgen withdrawal by suppressing ErbB3

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STATEMENT OF TRANSLATIONAL RELEVANCE

The goal of these studies is to identify therapeutic strategies that prolong the effectiveness of androgen withdrawal therapy (AWT) in patients with metastatic prostate cancer (PCa). Inhibitors of ErbB kinases such as erlotinib, lapatinib and trastuzumab have been tested in patients with castration resistant prostate cancer (CRPC) and in hormone-naïve patients, with little effect. Here we present novel data demonstrating that, instead, dual ErbB inhibitors sensitize PCa to AWT, and are thereby likely to prolong its effects. We show that during AWT, HER2 and ErbB3 levels increase, resulting in significant ErbB-dependent survival advantage that allows progression to CRPC. However, dual EGFR/HER2 inhibition, which inhibits their dimerization partner ErbB3 as well, induced apoptosis in cells undergoing AWT, despite ineffectiveness in hormone-naïve cells and in cells that have already progressed to CRPC. Our data indicate that administration of dual EGFR/HER2 inhibitors in PCa patients undergoing AWT may impede the onset of CRPC.

ABSTRACT

Purpose: Patients with recurrent prostate cancer (PCa) are commonly treated with androgen withdrawal therapy (AWT); however, almost all patients eventually progress to castration resistant prostate cancer (CRPC), indicating failure of AWT to eliminate androgen-sensitive PCa. The overall goal of these studies is to determine whether dual inhibition of the receptor tyrosine kinases EGFR and HER2 would prolong the effectiveness of this treatment in PCa.

Experimental Design: We used androgen-dependent LNCaP cells and its CRPC sublines LNCaP-AI and C4-2. Additional data were collected in pRNS-1-1 cells stably expressing a mutant androgen receptor (AR-T877A), and in nude mice harboring CWR22 tumors. Studies utilized EGFR inhibitors erlotinib and AG1478, and HER2 inhibitors trastuzumab and AG879.

Results: Dual EGFR/HER2 inhibition induced apoptosis selectively in androgen-sensitive PCa cells undergoing AWT, but not in the presence of androgens, or in CRPC cells. We show that AWT alone failed to induce significant apoptosis in androgen-dependent cells, due to AWT-induced increase in HER2 and ErbB3, which promoted survival by increasing Akt phosphorylation. AWT-induced ErbB3 stabilized the AR and stimulated PSA, while it was inactivated only by inhibition of both its dimerization partners EGFR and HER2 (PCa cells do not express ErbB4); but not the inhibition of any one receptor alone, explaining the success of dual EGFR/HER2 inhibition in sensitizing androgen-dependent cells to AWT. The effectiveness of the inhibitors in suppressing growth correlated with its ability to prevent Akt phosphorylation.

Conclusions: These studies indicate that dual EGFR/HER2 inhibition, administered together with AWT; sensitize PCa cells to apoptosis during AWT.

INTRODUCTION

Androgen withdrawal therapy (AWT) is currently the standard of care for men with advanced prostate cancer (PCa) (1); however, it was found that in most patients its effects typically last 18-24 months, after which the patient developed resistance to such therapy (castration resistant prostate cancer; CRPC). Although some groups reported increased apoptosis in prostatic tissue following AWT (2, 3), others found no increase in apoptotic indices in the majority of tumors (4, 5), although proliferation indices were consistently suppressed (2, 4). These studies, therefore, concluded that “*androgen deprivation may act through suppression rather than ablation of prostatic cancers*” (5, 6). These reports indicate that failure to undergo apoptosis during AWT maybe a major cause of resistance of PCa cells to this therapy. Surviving cells likely undergo growth arrest and lie dormant following AWT, but will revive when an alternate growth stimulant comes to release it from this growth arrest, as was demonstrated in a CWR22 xenograft model (7, 8). Therefore, adjuvant therapy that causes apoptosis during AWT would impede the onset of CRPC.

Here we investigate the role of ErbB inhibitors in this effect. The ErbB family of four closely related type 1 transmembrane tyrosine kinase receptors include the epidermal growth factor receptor (EGFR/HER1/ErbB1), and related family members ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4) (9). The ErbB receptors are activated by ligand binding, dimerization and phosphorylation. EGFR, ErbB3, ErbB4, but not HER2, have specific ligands, such as EGF for EGFR and heregulins (HRG1-4) for ErbB3 and ErbB4 (9). However, ErbB3 lacks significant kinase activity; hence both HER2 and ErbB3 require heterodimerization, with each other or the other ErbB receptors, for phosphorylation and activation. Significantly, PCa cells typically lack ErbB4 expression, but express high levels of ErbB3 (10, 11).

EGFR and HER2 are known to regulate cell proliferation, differentiation, angiogenesis and survival (12); however, in clinical trials for patients with CRPC, studies using selective and

specific inhibitors of individual receptors did not show any significant effect (13-17). In recent times, a number of dual EGFR/HER2 inhibitors have been developed, and were found to be more effective against PCa cells and animal models compared to the single inhibitors (18, 19). Tyrosine phosphorylation of HER2 and ErbB3, transactivation of the androgen receptor (AR), and cell proliferation induced by heregulin were more potently inhibited by the EGFR/HER2 dual tyrosine kinase inhibitor GW572016 (lapatinib) than the EGFR-specific inhibitor gefitinib (20, 21). Despite the success of the pre-clinical studies, in phase II single-agent clinical trials, lapatinib was fairly well-tolerated and resulted in stable disease for 12 weeks but evidenced no decrease in prostate specific antigen (PSA), an AR transcriptional target, in patients with hormone sensitive PCa (22) or in unselected patients with CRPC, as measured by PSA (23).

Here, we concentrate on the effects of dual EGFR/HER2 inhibitors and the conditions under which they are effective. It is known that AR function at low levels of androgen is mediated not by EGFR, but by the heterodimerization of HER2 with ErbB3 (18). Sergina et al demonstrated that ErbB3 was upregulated and provided compensatory signaling precisely in response to EGFR/HER2-directed tyrosine kinase inhibitor (TKI) treatment (24). Indeed, ErbB3-directed RNA inhibition duly restored the pro-apoptotic effects of TKIs (24). These reports suggested that the failure of EGFR and HER2 inhibitors may be due to the activation of ErbB3 in these tumors. Studies conducted *in vitro* (25, 26), in animal models (6), and in clinical specimens (27) indicate an increase in Akt phosphorylation during AWT which promotes cell survival. Based on these reports we investigated whether dual EGFR/HER2 inhibitors were effective when they downregulated ErbB3 and/or Akt phosphorylation, and whether they impede PCa progression to CRPC by inducing cell death during AWT.

MATERIALS AND METHODS

Cell Culture and Pharmacological Treatments Androgen-dependent LNCaP prostate cancer cells were purchased from American Type Culture Collection (ATCC, Manassas, VA), and C4-2 cells were obtained from UroCor (Oklahoma City, OK). Castration resistant clones of LNCaP cells (LNCaP-AI cells) have been described by us elsewhere (11, 25). pRNS-1-1 cells were also described earlier (11, 28). Recombinant human epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1) were obtained from Invitrogen, (Carlsbad, CA), recombinant human heregulin 1 (HRG1) was from PeproTech INC. (Rochy Hill, NJ). AG1478 and AG879 were from Calbiochem, EMD Chemicals, Inc. (Gibbstown, NJ). Erlotinib (Tarceva) was provided by OSI Pharmaceuticals, Inc. (Melville, NY), and also was obtained from LC Laboratories (Woburn, MA), while trastuzumab (Herceptin) was a gift from Genentech, Inc. (South San Francisco, CA). Bicalutamide (Casodex) was kindly provided by AstraZeneca (Cheshire, UK), while lapatinib was purchased from LC Laboratories (Woburn, MA). Rabbit polyclonal EGFR, HER2, ErbB3, β -actin and AR antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho-Akt (Ser 473), anti-phospho-EGFR (Y1068), anti-phospho-HER2 (Y1248), phospho-ErbB3 (Y1289), α -tubulin and Akt antibodies were from Cell Signaling Technology (Beverly, MA). Transfections and plasmids used have been described earlier (11). Human Akt1 siRNA was obtained from Santa Cruz Biotechnology, Santa Cruz, CA against the sequence: 5'-ACGAGGGGAGUACAUCAAGAC-3'.

Mouse Studies: 4-5-week old Balb/c athymic nude-Foxn1nu (nu/nu) male mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Suspensions of CWR22 cells were mixed in 50% Matrigel solubilized basement membrane (BD Biosciences, Bedford, MA) and xenografts were established by subcutaneous injections of 2.5×10^6 cells/site into the flanks. When palpable tumors were observed, animals were treated with (i) vehicle or (ii) a combination of erlotinib (0.8 mg/Kg, 100 μ l per dose, 5 times per week by oral gavage) and trastuzumab (20

mg/Kg, 90 μ l per dose, 2 times per week by i.p. injection), dissolved in a solution of phosphate buffered saline (PBS) and 0.5% Tween 20. 3 days after start of drug regimen, the animals were castrated by bilateral scrotal excision, following isoflurone-anesthetization. Control animals were sham-operated by opening the animals surgically, but no tissues were removed. Drug administration was continued post-surgery, but after 8 days, the mice were euthanized, tumors were collected and divided into sections for paraffin-embedding and snap-freezing in liquid nitrogen. Mice were weighed and blood was collected periodically and PSA levels measured by a standard ELISA kit (Fitzgerald Industries Intl., Acton, MA).

Immunohistochemistry and Statistical Analysis: We used rabbit polyclonal anti-ErbB3 (C-17) (1:100 dilution) antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, Ki67 was from DAKO (Carpinteria, CA), while TUNEL kit was from Millipore (Billerica, MA). For negative controls we used a Universal Rabbit IgG control (DAKO) in place of the primary antibody. Diaminobenzidine (DAB) was used as a chromogen, and counterstaining was with hematoxylin. Only the epithelial cells were scored. The extent of staining was scored 0-3, where 0 represented no staining, +0.5 represents low (<20% staining), +1 represent intermediate (30-50%), +1.5 (50-70%) and +2 represent high staining (>80%). To evaluate the differences in staining expression in the three diagnostic groups, we used t-tests with a Welch approximation. Columns represent the mean \pm standard deviation of samples from each group.

Flow cytometry and MTT assay: MTT and flow cytometric analysis was carried out as described earlier (11, 29, 30). Proliferation was estimated in propidium iodide stained ethanol-fixed cells by MODFIT (Verity software, Topsham, ME), while the rate of apoptosis induction was estimated in live cells staining with Annexin V by CellQuest V3.1 (Becton-Dickinson, Franklin Lanes, NJ).

RESULTS

Dual EGFR/HER2 inhibition sensitized androgen-dependent prostate cancer cells, but not castration resistant lines, to apoptosis by androgen withdrawal. We first compared the individual effects of the HER2 inhibitor trastuzumab (21 μ g/ml), and the EGFR inhibitor erlotinib (10 μ M), to dual inhibition with both drugs in androgen dependent LNCaP PCa cells. The drug combination caused cell cycle arrest in LNCaP cells following 48 hours of treatment in FBS medium (**Figure 1A, upper**). Culture in CSS, where androgen levels are significantly lower, also induced cell cycle arrest, but very little apoptosis, in these cells. However, the combination of trastuzumab and erlotinib, but not the individual drugs, induced 10-fold higher apoptosis in LNCaP cells in CSS-containing media (**Figure 1A, lower**). The overall effect is that, in FBS, dual EGFR/HER2 inhibition prevented cell number increase, whereas upon culture in CSS, additionally, there was a decrease in cell numbers indicating cell death (**Supplemental Figure 1A**). Unlike LNCaP cells, however, its CRPC sublines C4-2 (**Figure 1A, lower**) or LNCaP-AI (**Supplementary Figure 1B**), which have higher AR transcriptional activity (25), did not respond to dual inhibition of EGFR and HER2 even in CSS. Similarly, LNCaP cells underwent apoptosis in response to the dual EGFR/HER2 inhibitor lapatinib in CSS, but not in FBS, while its CRPC subline C4-2 cells were resistant to apoptosis by this drug (**Supplementary Figure 2**). Dual EGFR/HER2 inhibition prevented cell growth in FBS in AR-negative pRNS-1-1 cells stably transfected with vector only, but not those expressing AR(T877A), an androgen-sensitive active mutation found in LNCaP cells (**Figure 1B**). However, in CSS, where AR was inactive, this treatment inhibited growth, despite the presence of the AR(T877A) mutant (**Figure 1B**). These results indicate that AR activity suppresses the effects of ErbB inhibitors.

Androgen withdrawal stimulates, while dual EGFR/HER2 inhibition suppresses, ErbB3 levels. 48 hour treatment with erlotinib (10 μ M), but not trastuzumab (21 μ g/ml) inhibited EGF-

stimulated EGFR phosphorylation, whereas trastuzumab, but not erlotinib, affected the expression of HER2 (**Figure 2A, left**). On the other hand, the combination, but not the individual drugs, inhibited ErbB3 phosphorylation, and reduced ErbB3 levels (**Figure 2A, right**) also (**Supplementary Figure 3A**). Since PCa cells do not express ErbB4 (**Supplementary Figure 3B**) (10), we examined the effects of AWT on the levels of the other ErbB receptors. There was no significant change in EGFR levels upon culture in CSS, however, both HER2 and ErbB3 levels increased significantly as AR levels declined (**Figure 2B, upper panels**) (also **Supplementary Figure 3C**). Consistent with previous findings (6, 26), we saw a concomitant increase in Akt phosphorylation (Ser 473) in LNCaP (**Figure 2B, upper**). However, AWT caused no change in ErbB3 in LNCaP-AI cells, which expressed both higher AR (11) and ErbB3 (**Figure 2B, lower panels**). Comparison of LNCaP vs LNCaP-AI showed that the latter expressed higher levels of HER2 and ErbB3, and also higher ErbB3 phosphorylation (**Figure 2C**). Taken together, these results indicate that in LNCaP cells, but not its CRPC subline, ErbB3 levels increase during AWT whereas it is suppressed by dual EGFR/HER2 inhibition.

Dual EGFR/HER2 inhibition suppresses ErbB3 and PSA levels in CWR22 xenografts in nude mice. CWR22 xenografts were established in 4-5 month old male nude mice, and when the tumors were palpable, the animals were treated with vehicle only or with erlotinib (0.8 mg/Kg, 5 times per week) and trastuzumab (20 mg/Kg, 2 times per week) in combination. The animals were castrated, or sham operated, 3 days after the drugs were started, but drug treatments were continued until the end. The animals were divided as: (a) vehicle only, sham operated (n=6), (b) vehicle only, castrated (n=6) and (c) drug-treated, castrated (n=6). CWR22 tumors shrink rapidly following castration, hence to obtain sizable tumors that can be analyzed; the animals were sacrificed 8 days after the procedure. Serum levels of prostate specific antigen (PSA), a clinical indicator of AR activity in the prostate, were analyzed in blood drawn (i) at the

beginning of the study, (ii) on the day of castration/sham operation, and (iii) at the end of the study (**Figure 3A, upper**). In vehicle-treated, sham operated animals, PSA levels increased significantly with time ($p=0.049$), whereas in castrated animals, the change in PSA was not significant. In those treated with the drug combination, PSA levels decreased three-fold. At the end of the study, the difference between PSA levels from castrated animals that were vehicle treated (16.3 ± 8.3 ng/ml) vs drug treated (4.3 ± 3.2 ng/ml) was significant ($p=0.02$), whereas the difference between sham-operated (29.8 ± 7.9 ng/ml) vs control animals were not ($p>0.05$).

Staining for ErbB3 in the formalin-fixed and paraffin-embedded (FFPE) sections showed weak staining in the sham operated mice ($n=6$) whereas the castrated and vehicle treated mice showed strong staining ($n=6$), which was eliminated in the castrated mice treated with the drug combination ($n=5$; one of the tumors was too small for analysis) (**Figure 3B**). Quantitation of the staining levels showed a significant increase in ErbB3 levels from sham operated, vehicle treated (0.63 ± 0.43) to castrated, vehicle treated tumors (1.33 ± 0.26) ($p=0.009$), which was reduced 40% in tumors treated with the drugs in castrated animals (0.8 ± 0.45) ($p=0.05$) (**Figure 3C**). Castration suppressed proliferation and induced apoptosis in these animals, as indicated by Ki67 and TUNEL staining (**Supplementary Figure 4**), respectively, whereas both effects were enhanced by treatment with the drug combination (**Figure 3D**). These results confirm that dual EGFR/HER2 inhibition reduce ErbB3 levels and reduces serum PSA levels.

ErbB3 overexpression stabilizes androgen receptor levels and promotes castration resistant cell growth mediated by Akt. LNCaP cells overexpressing ErbB3 grew at a much faster rate compared to parental LNCaP cells (**Figure 4A, upper**) and were not growth inhibited by the AR antagonist bicalutamide (Casodex) even at 10 μ M (**Figure 4A, middle**) indicating androgen-independent cell growth. Flow cytometric analysis revealed this to be due to an increase in the percentage of cells entering the cell cycle (increased S-phase) which was not

impeded by bicalutamide (**Figure 4A, lower**). Although culture in CSS-containing medium causes a decrease in the levels of the AR in LNCaP cells, increased expression of ErbB3 in the same cells maintained AR levels (**Figure 4B**). Since ErbB3 is a known inducer of Akt phosphorylation (29), we examined the role of Akt in ErbB3-mediated cell growth. Increased ErbB3 stimulated Akt phosphorylation (**Figure 4C**), while downregulation of Akt expression by siRNA suppressed ErbB3-induced proliferation in LNCaP cells (**Figure 4D**), thereby indicating that Akt phosphorylation mediated the regulation of LNCaP cell growth by ErbB3.

Resistance to growth inhibition by dual EGFR/HER2 inhibition correlates with the ability of the inhibitors to suppress Akt phosphorylation. LNCaP-AI cells expressed higher levels of Akt phosphorylation compared to parental LNCaP cells (**Figure 5A, upper**). Treatment with the combination of trastuzumab and erlotinib, but not the individual drugs, significantly inhibited heregulin 1 β (HRG1)-induced Akt phosphorylation in LNCaP cells, but not in LNCaP-AI (**Figure 5A, lower**). Similarly, the same combination inhibited Akt phosphorylation in parental pRNS-1-1 cells which lack a functional AR, whereas in cells that express AR(T877A), the drug combination failed to inhibit Akt activity (**Supplementary Figure 5A**). These results correlate Akt phosphorylation with the growth inhibitory effects of the combination of trastuzumab and erlotinib. In addition, the tyrphostins AG1478 (EGFR inhibitor) and AG879 (HER2 inhibitor) (**Figure 5B, upper**), in combination, inhibited Akt phosphorylation in CSS-, but not in FBS-containing medium (**Figure 5B, lower**). Similar to trastuzumab and erlotinib, the combination of AG1478 and AG879, but not the individual drugs, suppressed growth of pRNS-1-1(ART877A) cells in CSS-containing medium, whereas they had little or no effect on cell growth in FBS-containing medium (**Figure 5C**). On the other hand, LNCaP-AI cells were not growth arrested by the latter combination (**Supplementary Figure 5B**). These results indicate that suppression of cell growth by the drug combination correlates with inhibition of Akt phosphorylation.

Suppression of Akt phosphorylation sensitizes castration resistant prostate cancer cells to dual EGFR/HER2 inhibition. Finally, we investigated methods of overcoming the resistance of PCa cells to ErbB inhibitors. Since LNCaP-AI are not sensitive to dual inhibition of EGFR and HER2, and expressed higher ErbB3 compared to LNCaP, we investigated whether the increase in ErbB3 contributed to this resistance. Similar to the effects of a combination of erlotinib and trastuzumab, the combination of AG1478 and AG879 impeded the increase in cell numbers but did not reduce them below initial levels in LNCaP cells cultured in FBS (**Figure 6A, upper**), indicating growth arrest but not cell death. However, when the same cells were cultured in CSS, there was a 50% decrease in cell numbers indicating cell death (**Figure 6A, lower**). On the other hand, culture in CSS failed to have a similar effect in LNCaP cells overexpressing ErbB3 (**Figure 6B**), indicating that ErbB3 increase induced resistance to this drug combination. In support of a role for Akt phosphorylation in this process, LNCaP cells cultured in CSS experienced increasing Akt phosphorylation over a period of 5 days when exposed to vehicle alone whereas when they were exposed to the combination of AG1478 and AG879, Akt phosphorylation was significantly impeded (**Figure 6C, upper**). On the other hand, in LNCaP-AI cells resistant to this drug combination (**Supplementary Figure 5B**), the increase in Akt phosphorylation in response to CSS exposure was not affected (**Figure 6C, lower**). The fact that Akt phosphorylation increased upon CSS treatment in LNCaP-AI cells whereas ErbB3 levels did not (**Figure 2B**) indicates that other factors also contribute to Akt phosphorylation in CRPC. Our results indicated that, failure of dual EGFR/HER2 inhibition to induce apoptosis resulted from a failure of the same drugs to downregulate Akt phosphorylation. In support, AG1478 and AG879 in combination was not effective in inducing apoptosis in LNCaP-AI cells in the presence of control siRNA (9.89% in control siRNA vs 13.25% in control siRNA + AG1478 + AG879), whereas Akt siRNA alone induced a significant increase in Annexin V staining (28.28%) which was further increased in the presence of the drugs (44.65%) (**Figure 6D**).

DISCUSSION

Previous studies showed that the dual EGFR/HER2 inhibitor lapatinib evidenced no decrease in PSA in patients with hormone sensitive PCa (22) or in unselected patients with CRPC (23). The goal of this study was to determine whether dual EGFR/HER2 inhibition has any role in the prevention of disease progression in PCa. We demonstrate that androgen-dependent PCa cells with low ErbB activity do not show substantial response to ErbB inhibitors, whereas during AWT, ErbB2 and ErbB3 levels increase, which regulates Akt phosphorylation and also cell survival. Hence, during this period, if the increase in these receptors is inhibited by dual EGFR/ErbB2 inhibition, which also inhibits ErbB3 phosphorylation, the increase in Akt phosphorylation and survival can be prevented. However, once ErbB3 levels have increased, the same drugs fail to affect the levels of Akt phosphorylation, thereby indicating that they can inhibit *de novo* activation of ErbB3 but cannot dephosphorylate the receptor after it is activated.

Although individual EGFR and HER2 inhibitors had differential effects on PCa cells, the overall effect of dual inhibition was similar. The difference between various inhibitors of the same receptor may be attributed to the strength of the binding of these inhibitors to the receptor. We see that in both cases, the drug combinations resulted in a decrease in Akt phosphorylation. Since ErbB4 is lost in PCa, the ErbB dimers formed in this disease include EGFR homodimers and EGFR-HER2, HER2-ErbB3 and EGFR-ErbB3 heterodimers (discussed in details in (31)). All contribute to survival of PCa cells; hence inhibition of only one receptor will not prevent downstream signaling. Our data shows that inhibition of both EGFR and HER2 is required to prevent ErbB3 signaling, likely by preventing its dimerization. Since only ErbB3 but not EGFR or HER2 have p85 PI3K binding sites (9), the majority of the Akt signaling may be downstream of ErbB3 dimerization with EGFR or HER2, which will be inhibited only upon dual inhibition. ErbB3 monoclonal antibodies such as MM-121 are currently in development (32), and are also likely to succeed in combination with other ErbB inhibitors such as lapatinib.

We show that in cells expressing high AR, either hormone-naïve cells never exposed to AWT, or in CRPC cells that have high AR transcriptional activity, dual ErbB inhibition is unable to inhibit Akt phosphorylation and cell survival. In a previous study, we had shown that in hormone-naïve cells, the AR suppresses ErbB3 levels by transcriptionally regulating the ErbB3 inhibitor Nr4a1 (11). Since ErbB3 is capable of inducing AR-independent cell growth, this is likely an attempt by the AR to suppress AR-independent signaling. Hence, in androgen-dependent cells growing in the presence of high androgen levels, cell-survival is AR-dependent and not ErbB3-dependent. Therefore, inhibition of ErbB3 or its binding partners will not affect cell growth or survival. On the other hand, when AR levels decreased during AWT, ErbB3 levels rebound and cell growth becomes dependent on signal transduction downstream of this receptor. Therefore, if at this time, ErbB3 signaling is suppressed, cell survival is impacted.

ErbB3 increase during AWT likely as an attempt to prevent AR decrease. In this study, we show that ErbB3 stabilize AR levels; thereby preventing its decrease in low-androgen medium. Further studies are required to see whether this is the mechanism by which ErbB3 promotes androgen-independent cell growth, but if so, it will explain why, in some CRPC cells, growth is still AR dependent, but not androgen dependent, as has been demonstrated by other labs (33, 34). Despite this, it appears that the ErbB3-stabilized AR is incapable of downregulating ErbB3 (which is reasonable, if it requires that ErbB3 to stabilize it), as we previously showed (11). Furthermore, once the cell progresses to a CRPC phenotype, it is no longer capable of responding to dual EGFR/HER2 inhibition to downregulate Akt phosphorylation downstream of ErbB3. Hence, dual EGFR/HER2 inhibition does not affect cell survival or even cell growth in CRPC cells.

In CRPC cells, the effects of ErbB receptors and the AR are compounded by high Akt phosphorylation (29). Akt is induced by other factors including IGF, hence in CRPC cells, which are associated with multiple changes in cell signaling pathways (see (35) and references

within), it is likely that the cells have become adept at kinase switching, resulting in activation of multiple cell survival pathways. As a result, in these cells, dual EGFR/HER2 inhibition will not prevent all aberrant Akt phosphorylation. Therefore, our goal is to prevent the increase in aberrant Akt phosphorylation, and PSA progression, indicative of relapse, following AWT, by using the dual inhibitors during and not after this treatment. The clinical and therapeutic consequences of such a treatment could be quite profound. A 2009 study of 1,078 patients with hormone-sensitive PCa enrolled in SWOG trial 9346, where PSA progression (PSA-P) was defined as an increase of $\geq 25\%$ over nadir, median subsequent overall survival was shown to be 10 months in patients experiencing PSA-P within 7 months of hormone treatment, vs 44 months for those who did not have PSA-P during this period (36). Therefore, it is likely that if co-administration of dual EGFR/HER2 inhibitors delays PSA-P beyond 7 months, we would see a significant increase in survival.

In conclusion, our data indicate that dual EGFR/HER2 inhibition is an effective tool for sensitizing androgen-dependent PCa cells to apoptosis during AWT, likely preventing PCa progression to CRPC following AWT treatment, but is not effective in CRPC cells expressing high Akt phosphorylation. However, this strategy may find utility with the advent of new therapeutic agents such as abiraterone acetate, a CYP17 inhibitor that blocks steroid biosynthesis (37), and MDV3100, a more potent AR inhibitor (38). In post-docetaxel patients, abiraterone increased survival by 3.9 months over controls (37) and it would be of interest to see whether this leads to an increase in ErbB3/HER2 as well, and whether prevention of this increase, if any, would further prolong survival. It is clear from the current study, that the window of opportunity for using ErbB inhibitors in PCa is when ErbB3 is rising and not when it is stable. The study also demonstrates that potentially effective drugs if utilized in the wrong clinical setting may be prematurely judged to be ineffective.

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FIGURES AND LEGENDS:

FIGURE 1. Androgen withdrawal sensitized prostate epithelial cells to apoptosis by the combination of trastuzumab and erlotinib. (A) LNCaP cells were cultured in the presence of FBS or CSS for 48 hours together with trastuzumab (21µg/ml), erlotinib (10µM), or combinations thereof. The cells were collected and analyzed by flow cytometry to determine **(upper)** the fraction of cells in S-phase (which indicates proliferation) and **(lower)** those undergoing apoptosis (data presented represent fold changes over control cells treated with DMSO alone, 1.1% in LNCaP 3.48% in C4-2 cells). **(B) (upper panel)** MTT assay was used to determine the cell growth rate of parental pRNS-1-1 cells with the combination of Erlotinib (10 µM) and/or Trastuzumab (21 µg/ml) for 24 hours. **(Lower panels)** MTT assay to determine the effect of Erlotinib and/or Trastuzumab in the presence of **(middle panel)** medium containing FBS **(lower panel)** or CSS in pRNS-1-1 cells transfected with mutant AR (T877A). Data represents mean ± S.D. for three independent experiments.

FIGURE 2. ErbB3 inhibition by the combination of erlotinib and Trastuzumab, and its stimulation by AWT, in LNCaP cells. (A) Western blots demonstrating the effect of erlotinib (10 µM) and Trastuzumab (21 µg/ml) on ErbB receptor tyrosine kinases. **(left)** LNCaP cells were serum starved in the presence of erlotinib and/or trastuzumab for 48 hours, followed by further treatment with 10 ng/ml EGF for 5 mins. Lysates were blotted with anti-phospho EGFR (Y1068) **(1st Panel)**, anti-EGFR **(2nd Panel)**, or anti-HER2 **(3rd Panel)** antibodies. **(right)** Alternately, the cells were stimulated with 50 ng/ml HRG1 to induce ErbB3 phosphorylation, and immunoblotted with anti-phospho ErbB3 **(1st panel)** and anti-ErbB3 **(2nd panel)**. **(B)** Western blots demonstrating that AWT causes increased HER2 and ErbB3 expression and phosphorylation of Akt. LNCaP cells were cultured in FBS-containing medium up to 75% confluence and then switched to CSS-containing medium for the indicated period of time. Cell

lysate was collected and immunoblotted with antibodies to anti-AR (**1st panel**), anti-EGFR (**2nd Panel**), anti-HER2 (**3rd panel**), anti-ErbB3 (**4th Panel**), anti-phospho Akt (Ser 473) (**5th panel**), anti-Akt (**6th Panel**), and anti- β -actin (**7th Panel**). In contrast to LNCaP, it's CRPC subline LNCaP-AI did not experience a similar increase in ErbB3 following AWT (**8th panel**). **(C)** Comparison of the activation and expression of the ErbB receptors expressed in LNCaP cells and it's CRPC subline LNCaP-AI. The cells were serum starved for 48 hours and then EGF (10 ng/ml) (**left**), or HRG (50 ng/ml) (**right**) were added for the times indicated.

FIGURE 3 The combination of EGFR and HER2 inhibitors inhibited PSA and ErbB3 levels in CWR22 xenograft-bearing nude mice. (A) (upper panel) Serum PSA was measured in weekly blood draws from the three groups of animals: sham operated/vehicle treated (n=6), castrated/vehicle treated (n=6) and castrated/drug treated (n=5), **(lower panel)** while their body weight was monitored to determine overall health. **(B)** Representative ErbB3 stainings of tumors extracted from **(upper)** sham operated (this section was scored +1), **(middle)** castrated/vehicle treated (this section scored +2) and **(lower)** castrated/trastuzumab+erlotinib treated (this section scored +0.5) mice (20X). **(C)** The scores from each group were statistically analyzed to determine overall effects. Castrated/ vehicle treated mice had a significant overall increase in mean ErbB3 levels (1.33 ± 0.26 , n=6) compared to sham operated animals (0.63 ± 0.43 , n=6), $p=0.009$; which decreased again (0.8 ± 0.45 , n=5) in castrated/ drug treated mice ($p=0.05$). **(D)** Ki67 and TUNEL staining to determine levels of proliferation and apoptosis in CWR22 xenograft tumors in the same three groups. There was a significant decrease in nuclear staining for both Ki67 ($p=0.0027$) and TUNEL ($p=0.0037$) in cells from tumors extracted from the castrated+trastuzumab+erlotinib group compared to the sham castrated (intact) group (n=6).

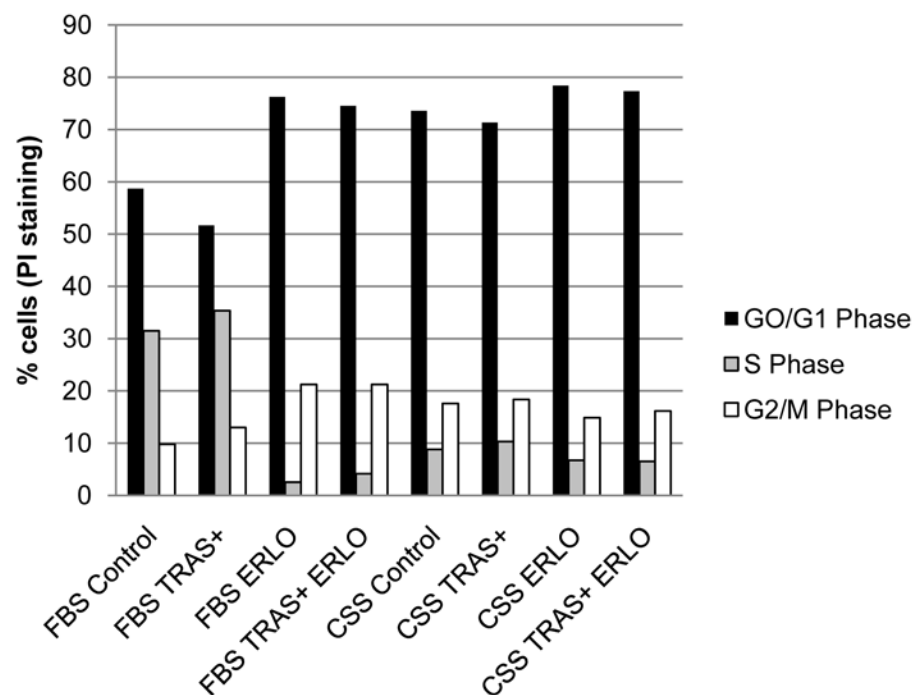
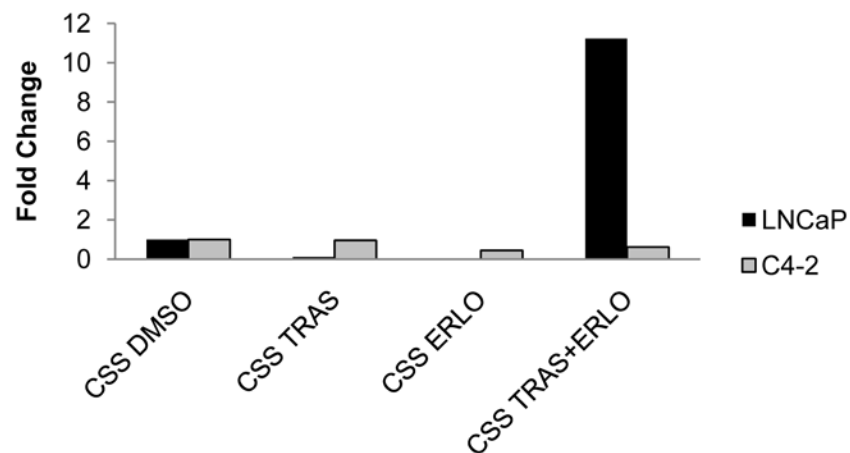
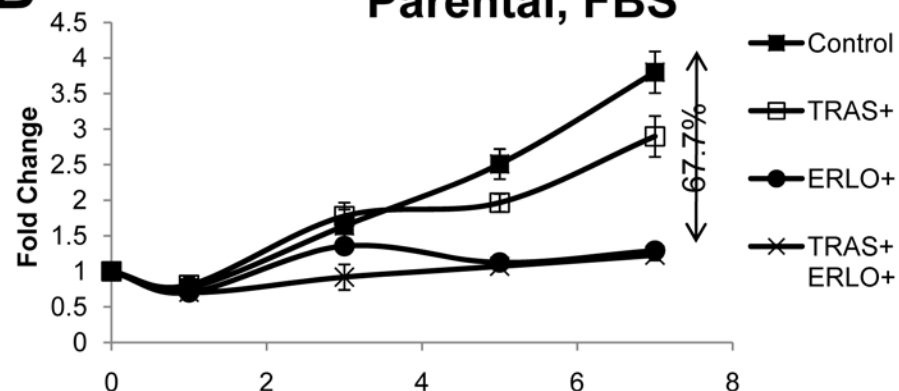
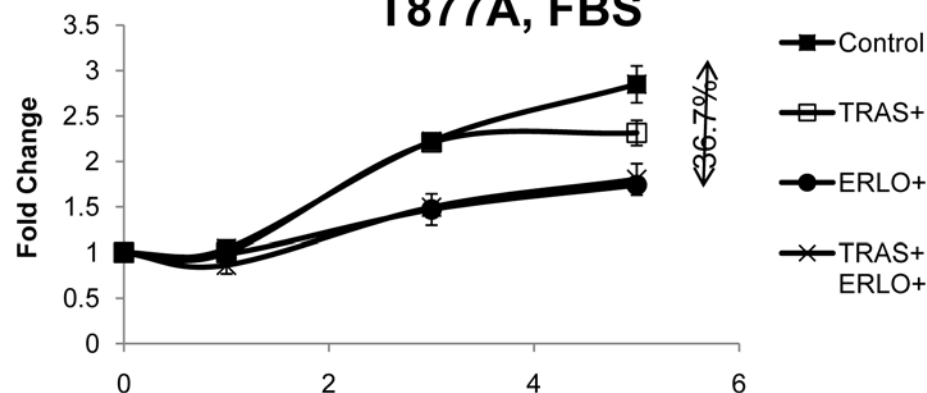
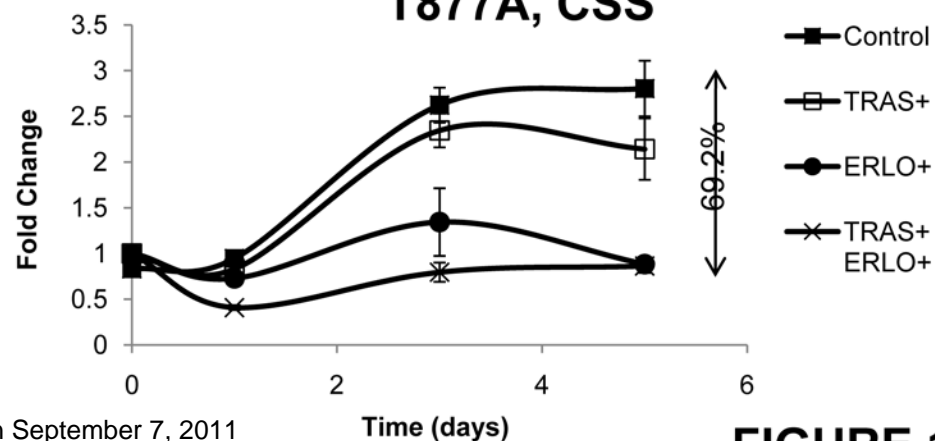
FIGURE 4. Increased ErbB3 levels induce castration resistant cell growth mediated by Akt and androgen receptor stabilization. (A) (upper) MTT assay showing the growth of LNCaP cells transfected with an empty vector or with pcDNA3-ErbB3 cultured in FBS over 6 days. **(middle)** MTT assay showing the growth rate of LNCaP-ErbB3 cells cultured with DMSO (control) or 10 μ M bicalutamide (Casodex). All data in this series is representative of three independent experiments. **(lower)** Flow cytometric analysis showing that LNCaP cells expressing pcDNA3 alone were responsive to bicalutamide-induced growth arrest whereas those expressing high ErbB3 levels did not. “*”: $p < 0.05$. **(B)** LNCaP cells transfected with vector (pCDNA3) or overexpressing ErbB3 were cultured in FBS-containing medium until 70% confluent, then switched to medium containing CSS and collected after the periods shown. AR and tubulin (loading control) levels were determined by Western blotting. **(C)** LNCaP cells were stably transfected with vector alone or with a plasmid expressing ErbB3, and demonstrates an increase in ErbB3 levels in the latter cells as well as an increase in Akt phosphorylation. **(D)** ErbB3 mediated cell growth was dependent on Akt activation. LNCaP cells transfected with vector alone or ErbB3 plasmid were subjected to treatment with control or Akt siRNA. The effect of Akt siRNA on Akt levels are shown in the inset. Growth rates were estimated after 4 days of treatment by MTT assay.

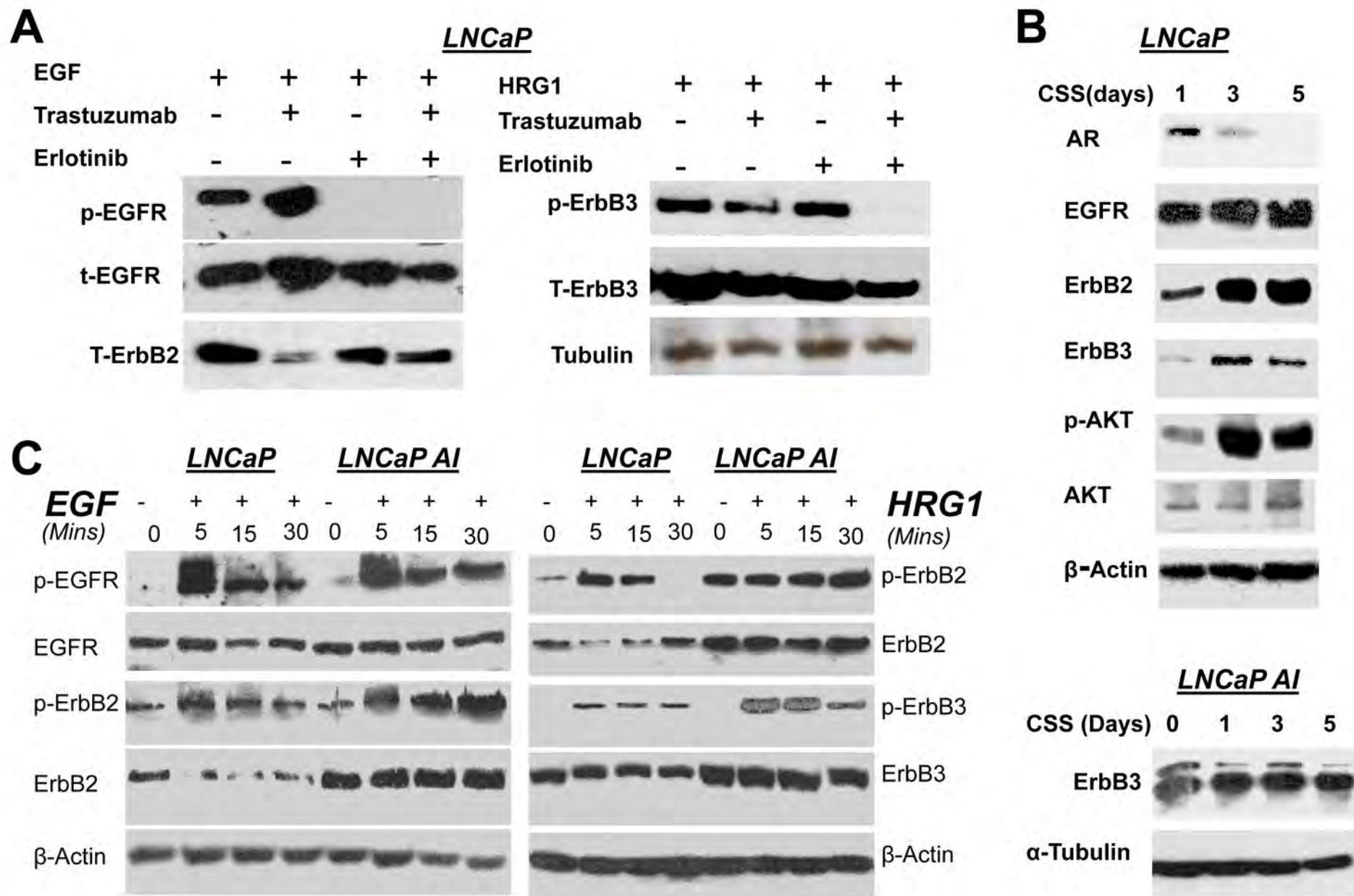
FIGURE 5. Akt phosphorylation at Ser 473 correlates with the ability of ErbB inhibitors to impede cell growth. (A) (upper) LNCaP-AI cells experience increased levels of Akt phosphorylation (Ser 473) compared to LNCaP. LNCaP-AI cells were serum starved and then treated with 10 ng/ml IGF-1 for various times as shown. Note the increase in Akt phosphorylation at Ser 473 with time. **(lower)** Western blots demonstrating the effect of erlotinib (10 μ M) and trastuzumab (21 μ g/ml) on LNCaP and LNCaP-AI cells. Cells were grown to 75% confluence, and then serum starved for 48 hours in the presence of erlotinib or trastuzumab or

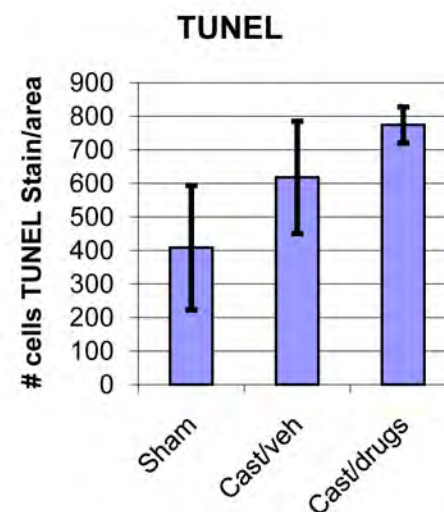
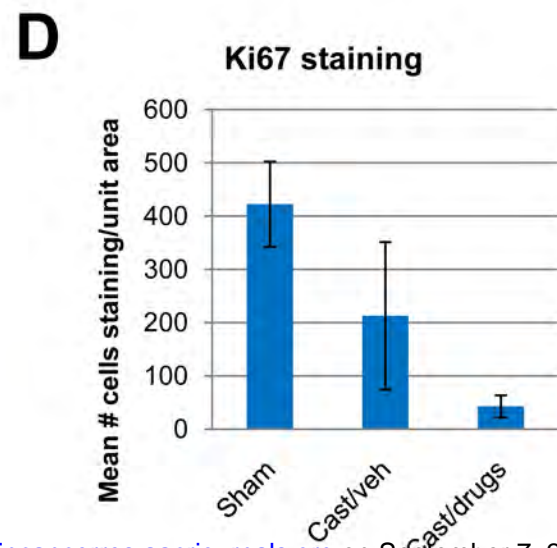
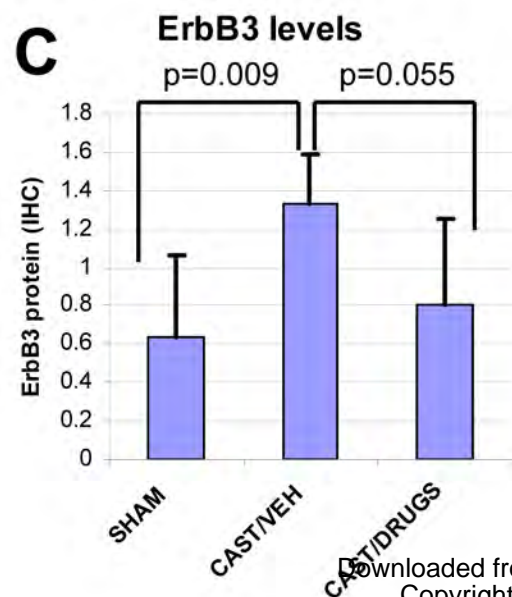
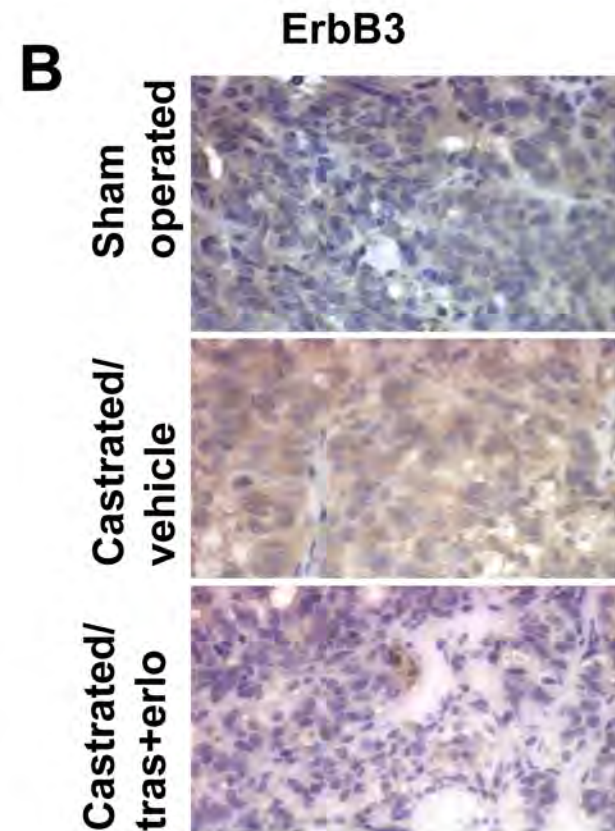
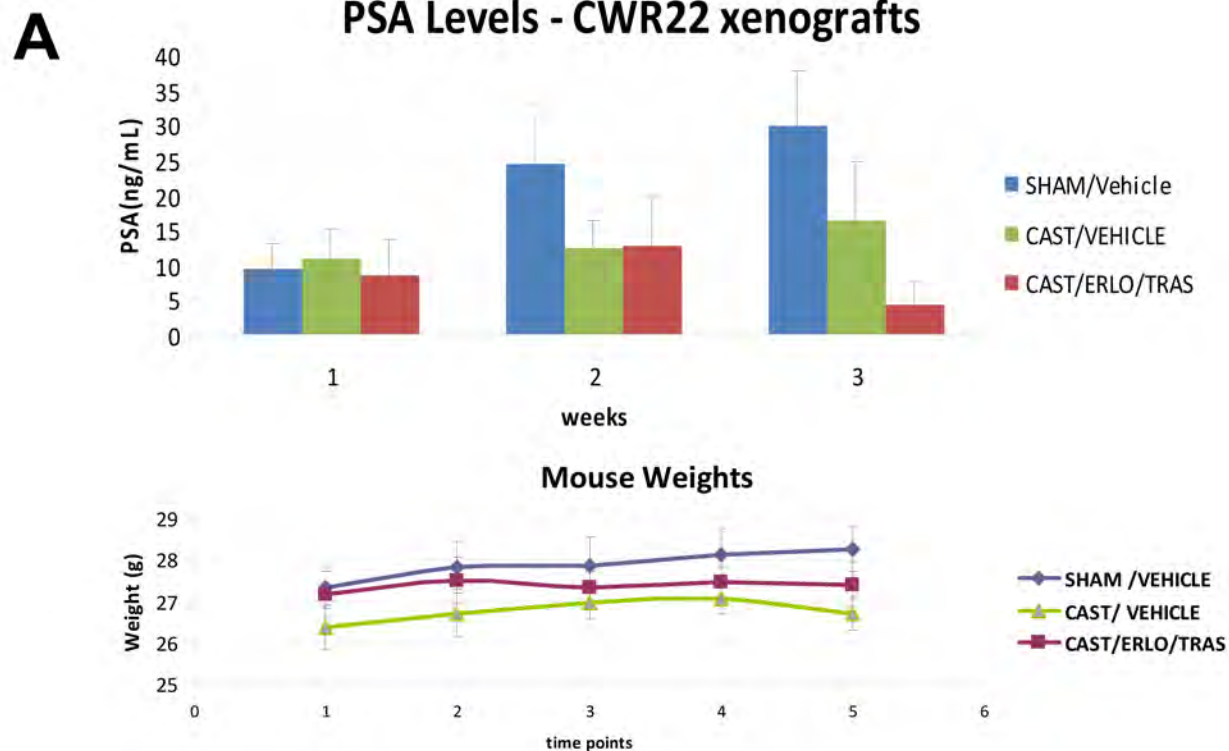
both. The cells were then further treated with 50 ng/ml HRG1 for 15 mins, to stimulate Akt phosphorylation downstream of ErbB3 activation, cell lysates collected and immunoblotted with antibodies to anti-phospho Akt (Ser 473) (**1st, 3rd Panels**), and total Akt (**2nd, 4th Panels**). **(B) (upper)** Western blots demonstrating the specificity and selectivity of AG1478 and AG879 on the activation of EGFR and HER2 respectively. Serum starved LNCaP cells were treated with vehicle (DMSO), 5 μ M AG1478 or 2 μ M AG879 for 48 hours followed by further treatment with PBS or 10 ng/mls EGF for 5 mins. EGF induced the phosphorylation of both EGFR (Tyr1068) and HER2 (Tyr1248). **(lower)** LNCaP cells cultured in FBS or CSS were treated with the two drugs for 3 or 5 days. Western blotting shows that in the presence of FBS, there was no effect of the drugs, alone or in combination, on Akt phosphorylation whereas in CSS, Akt phosphorylation at Ser 473 was significantly affected. **(C)** MTT assay was used to determine the cell growth rate with the combination of AG879 (2 μ M) and AG1478 (5 μ M) of pRNS1-1 cells stably transfected with a T877A mutant AR grown in medium containing FBS (**left panel**) or medium containing CSS (**right panel**). Data represents mean \pm S.D. of three independent experiments.

FIGURE 6. ErbB3 overexpression induces resistance to dual EGFR/HER2 inhibition in CRPC, which can be overcome by Akt downregulation. (A,B) LNCaP cells expressing vector alone, or overexpressing erbB3, were treated with 2 μ M AG879, 5 μ M AG1478, or both, were cultured in medium containing FBS or CSS. MTT assays were conducted to determine the effects of the drug combination on cell growth. **(A, upper)** In medium containing FBS, where control cells experienced a 2.25-fold increase in cell number after four days of treatment, those treated with a combination of AG1478 and AG879 failed to grow ($p < 0.0001$), but showed no decrease in cell numbers. **(A, lower)** LNCaP cells transfected with vector only showed a decrease in cell numbers upon culture in CSS. **(B)** FBS- or CSS-cultured, ErbB3-transfected

LNCaP cells demonstrated comparable increase in growth rates (2.5-fold increase in growth in 4 days), but dual treatment with AG1478 and AG879 prevented growth ($p=0.004$), but did not decrease cell numbers. Data represents mean \pm S.D. of three independent experiments for each point. **(C)** LNCaP and LNCaP AI cells were cultured in FBS then switched to CSS-containing medium in the presence of vehicle (DMSO) or a combination of AG1478 and/or AG879. Cells were harvested after the indicated period of time, and cell lysates run on 10% SDS-PAGE, immunoblotted and the blots stained with rabbit polyclonal anti-phospho-Akt (Ser 473) antibody. **(D)** Flow cytometric analysis of LNCaP-AI cells following 48 hour treatment with siRNA duplexes against a scrambled sequence or Akt1 siRNA. Propidium iodide and Annexin V-FITC stained cells were then analyzed by flow cytometry to determine the fraction of cells undergoing apoptosis.

A**LNCaP****Annexin V stain****B****Parental, FBS****T877A, FBS****T877A, CSS**





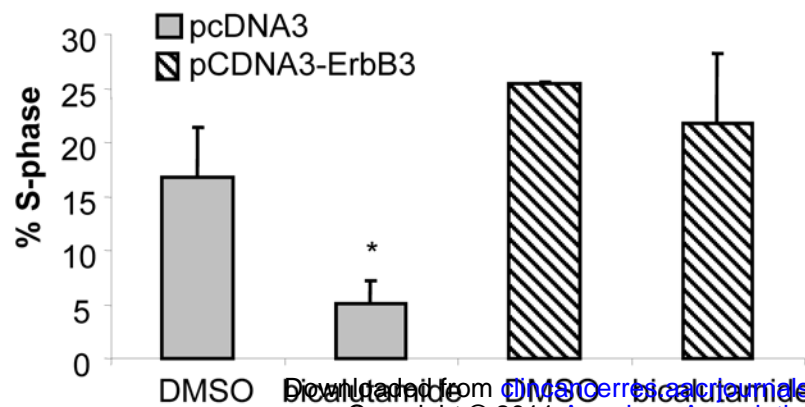
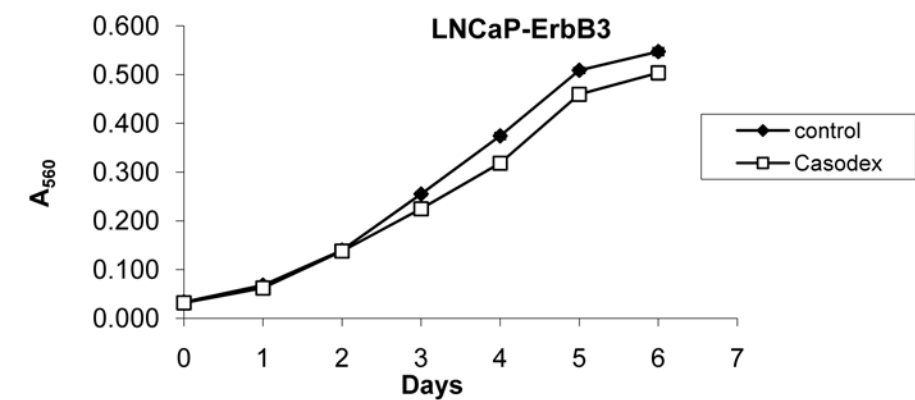
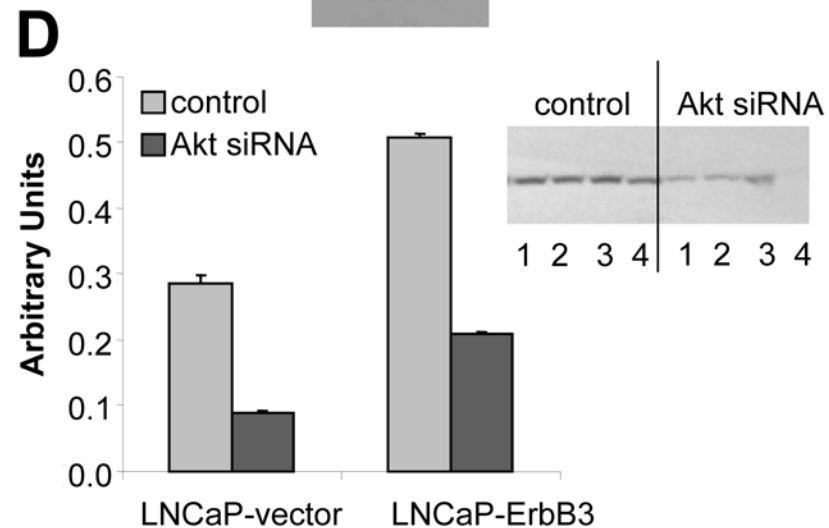
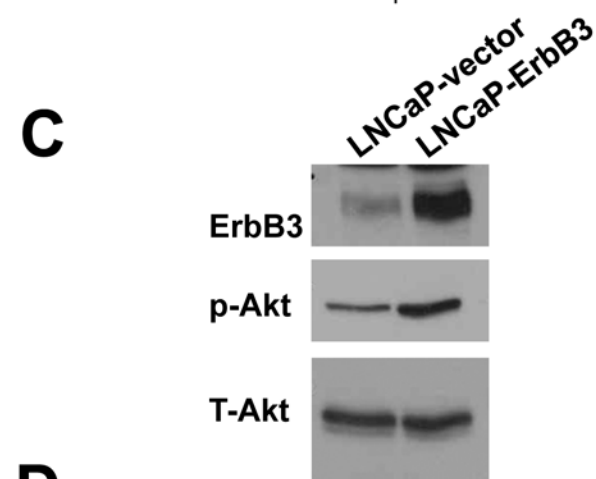
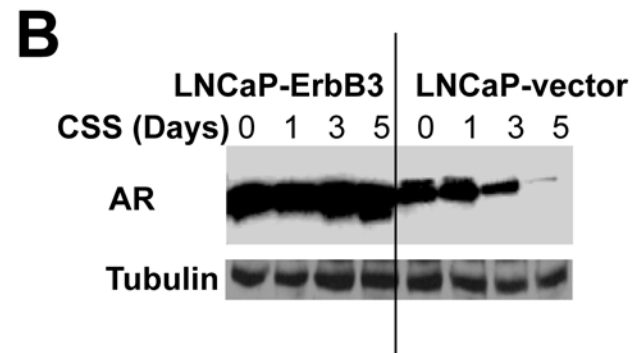
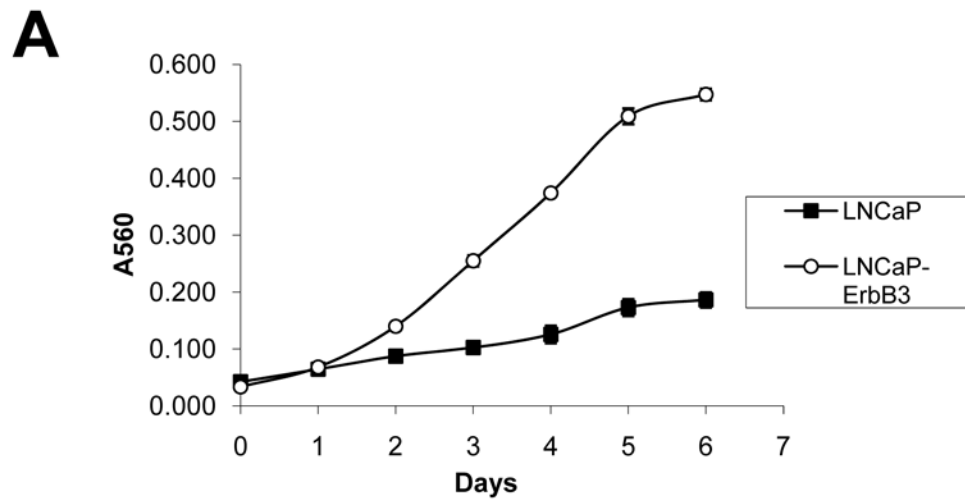


FIGURE 4

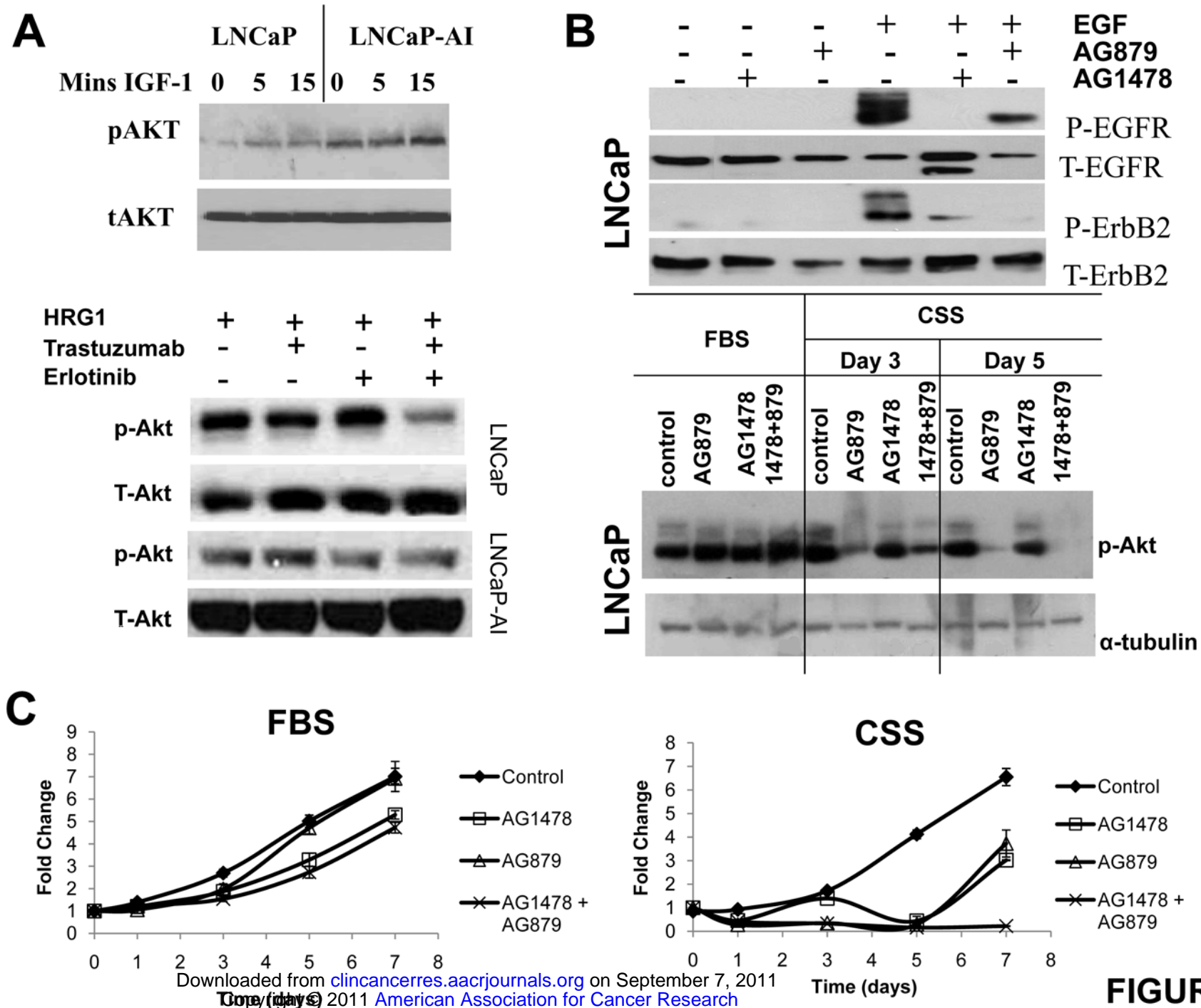


FIGURE 5

